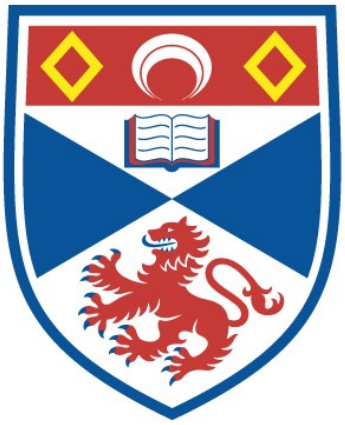
CARBON DIOXIDE FIXATION IN BARLEY ROOTS, WITH SPECIAL REFERENCE TO ITS RELATIONSHIP TO MINERAL ION ABSORPTION

Janet Scott Douglas Graham

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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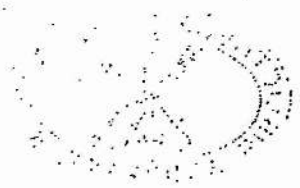

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Carbon dioxide fixation in barley roots, with special reference to its relationship to mineral ion absorption.

by

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A Thesis submitted to the University of St. Andrews for the Degree of Doctor of Philosophy.

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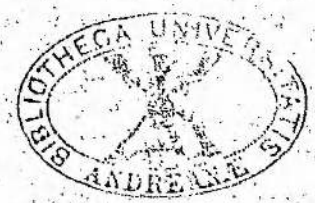

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Declaration.

I hereby declare that the following Thesis is based on the record of work done by me, that the Thesis is my own composition, and that it has not previously been presented for a Higher Degree.

The research was carried out in the Department of Botany at St. Salvator's College of the University of St. Andrews under the direction of Dr. L. W. Poel.

Certificate.

I certify that Janet Scott Douglas Graham has spent nine terms of Research Work under my direction, and that she has fulfilled the conditions of Ordinance No. 16 (St. Andrews), and that she is qualified to submit the accompanying Thesis in application for the degree of Doctor of Philosophy.

Career.

I first matriculated in the University of St. Andrews in October 1948, and was elected to an Exchange Scholarship to Sweet Briar College, Virginia, U.S.A., for the academic session 1950-51. I graduated Ordinary B.Sc. in October 1951, and added Honours of the First Class in June 1953.

In October 1953 I was admitted as a Research Student in the University of St. Andrews under Ordinances 16 and 61, and was awarded a Research Scholarship from the Carnegie Trust to the Universities of Scotland, which I held until April 1956.

Acknowledgments.

I wish to record my deep appreciation to Dr. L. W. Poel of the Department of Botany, St. Salvator's College, for supervising the work presented in this Thesis, and for the stimulating interest he has shown throughout the investigation.

I am also indebted to Mr. A. Patrick of the Department of Botany, St. Salvator's College, for constructing the chromatography cabinet and boxes, and the drying oven, and for his assistance to Dr. L. W. Poel in constructing the pen-recorder.

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Finally, I am also grateful to Professor J. H. Burnett of the Department of Botany, St. Salvator's College, and to Dr. L. W. Poel for their criticisms of the manuscript.

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1. Introduction.

For many years it was believed that carbon dioxide fixation occurred only in photosynthetic and chemosynthetic autotrophic organisms. Wood and Werkman (1936), in their experiments with propionic bacteria, were the first to demonstrate carbon dioxide utilization by a heterotrophic organism. Subsequent investigations in both plant and animal tissues have shown that the ability to fix carbon dioxide is possessed by many, probably all, forms of life (Utter and Wood, 1951).

The use of isotopes has greatly facilitated the study of both photosynthetic and heterotrophic carbon dioxide uptake, and has enabled both qualitative and quantitative analyses to be made. The short-lived radioisotope carbon-11 was used in the early investigations on carbon dioxide fixation in bacteria (Barker, Ruben and Beck, 1940; Barker, Ruben and Kamen, 1940; Carson and Ruben, 1940), yeast (Ruben and Kamen, 1940) and in the fungi (Foster et al, 1941). The rapid decay of this isotope seriously limits its use as a tracer, particularly in determining the nature of the fixation products.

Carbon-13, a stable isotope, has been used in various investigations; Wood et al (1941) studied carbon-13 assimilation in bacteria, and Slade et al (1942), also using carbon-13, concluded that carbon dioxide assimilation was a general

phenomenon among heterotrophic bacteria. Utter and Wood (1946) demonstrated carbon-13 dioxide uptake in pigeon liver with the formation of labelled oxalacetate.

Carbon-14 became much more readily available after 1945, and since then it has had a widespread application in biological research; with a half-life of over 5000 years, it has completely superseded carbon-11 in tracer experiments. It has been used for investigations on carbon dioxide fixation in bacteria (Barker and Kamen, 1945; Tomlinson, 1954), fungi (Foster et al, 1949; Martin and Wilson, 1951; Heplar and Tatum, 1954); and in animal tissues (Crane and Ball, 1950; Goldberg and Sanadi, 1952; Marshall et al, 1954).

The ability of the leaves of higher plants to incorporate carbon dioxide in the dark has been observed, although the rate of uptake is only a small percentage of that occurring in the light (Calvin et al, 1952; Nezgoborodba, 1952). The products of fixation are those carboxylic and amino acids which are closely related to the Krebs tricarboxylic acid cycle (Benson and Calvin, 1950; Lynch and Calvin, 1952), although in the dark fixation of various leaves, Nezgoborodba (1952) observed that the maximum fixation of the isotope was found in the protein fraction.

The dark carbon dioxide fixation assumes considerable proportions in the case of succulent plants, carbon dioxide being a metabolite in acid synthesis (Thomas, 1952; Ranson, 1953).

Bryophyllum leaves after 60 hours dark exposure had incorporated carbon-14 not only into the organic acids, but also into proteins and carbohydrates (Thomas, 1952) indicating the wide utilization of the fixed carbon. Heterotrophic assimilation of carbon dioxide leading to the synthesis of cellular material was also observed in bacteria (Barker, Ruben and Kamen, 1940) and in moulds (Foster et al, 1941). The similarity of the products in both heterotrophic and dark carbon dioxide fixation by photosynthetic tissues, suggests that the mechanism of autotrophic fixation may resemble in certain aspects the heterotrophic fixation of carbon dioxide (Wood and Lorber, 1949; Lynch and Calvin, 1952).

A generalised equation for carbon dioxide utilization was proposed by Van Niel et al (1942):-

$$RH + CO_2 \leftrightarrows R.COOH$$

and the main difference between heterotrophic and autotrophic fixation is that in the former the hydrogen for reduction is derived by oxidation of organic materials (Ochoa, 1951, 1952). This reversibility of the oxidative decarboxylation of dicarboxylic and tricarboxylic acids has an important role in the maintenance and regulation of these metabolites, controlling thereby the respiratory metabolism of the cell (Ochoa, 1952). Furthermore, it has been shown that carbon dioxide is an essential metabolite for growth in many organisms

(Ruben and Kamen, 1940; Van Niel et al, 1942). Gladstone et al (1935) found that the presence of carbon dioxide in the medium was a prerequisite for the growth of all bacteria examined, and similarly Hes (1938) observed that in the absence of carbon dioxide all growth was suppressed in bacteria, yeast, and moulds. But growth of bacteria, in the absence of any external source of carbon dioxide, was stimulated if dicarboxylic acids were supplied (Herbert, 1952).

Heterotrophic carbon dioxide fixation in roots was first demonstrated by Ruben and Kamen (1940) in a preparation of ground barley roots exposed to carbon-11, and the accumulation of radioactive carbon due to absorption of labelled bicarbonate ions was investigated by Overstreet et al (1940). Evidence of carboxylation reactions resulting in the incorporation of carbon-14 dioxide into both dicarboxylic and tricarboxylic acids was shown in parsley root extracts (Gollub and Vennesland, 1947; Vennesland et al, 1947; Coitham1 and Vennesland, 1949). Barley roots exposed to carbon-14 dioxide when inhibited by malonate accumulated labelled succinate (Laties, 1947). This indicates an interrelationship of the tricarboxylic acid cycle and carbon dioxide assimilation.

Investigations on the uptake of carbon-14, in the form of carbonate and bicarbonate solutions, by roots of

intact Phaseolus vulgaris seedlings (Kursanov et al, 1951), and as gaseous carbon dioxide by excised and intact roots of Phaseolus vulgaris and Primula obconica (Kuzin et al, 1952) showed a small translocation of the labelled products to the green parts of the plant. The isotope was mainly incorporated into the organic acid fraction but some activity was also present in both the carbohydrate and protein fractions. Analysis of the organic acid fraction of young bean roots showed that the carbon-14 was mostly in malate (Kursanov et al, 1953). The presence of mycorrhiza in the roots of Calluna vulgaris had no apparent qualitative effect on the products of carbon dioxide fixation (Pryde, 1955), as the labelled compounds formed were similar to those obtained in barley roots (Pool, 1952, 1953).

Pool (1952,1953) investigated the effect of various factors on carbon dioxide uptake by excised barley roots. Experiments to demonstrate the rate of carbon dioxide fixation showed that most of the carbon dioxide assimilation occurred within the first ten minutes of the exposure period. Under anaerobic conditions uptake of carbon-14 dioxide was reduced to a very low level. The effect of pretreatment in mineral salt solution as compared to that in distilled water or tap water, had a marked influence on the subsequent fixation of carbon-14 dioxide in the presence of phosphate

buffer. The presence of mineral ions during pretreatment reduced the fixation to about 20% of that occurring with distilled water, and this decrease was mainly reflected in the malic acid fraction.

The major purpose of the present investigation has been to elucidate the relationship between mineral ion absorption and carbon dioxide fixation, and to determine whether the observed reduction after mineral ion pretreatment was conditioned by the presence of a specific mineral element or if it was the result of mineral ion absorption as a whole. Jacobson (1955) has also worked on this aspect, and has studied the uptake of carbon-14 dioxide by barley roots during exposure to single salt solutions, providing different ratios of cation : anion absorption.

II. General methods.

A. Plant material.

The Californian variety of barley 'Atlas 46' was used, which was supplied by Dr. Arnon in 1951, and by Dr. Schaller in 1954 from the University of California. Seed (50 gms.) was soaked for about 6 hours in unaerated tap water or distilled water, and was then sown on cotton netting supported on two glass frames (20 x 22 cm.). These were placed over enamel dishes (25 x 20 cm.) in such a way that the netting dipped into the culture medium on either side. In the initial experiments the medium (approximately 1000 ml.) was tap water, but owing to the high mineral content of the tap water, this was subsequently changed to distilled water. The absence of nutrient solution during germination and growth of the seedlings, provided roots with a high capacity for salt absorption (Prevot and Steward, 1936).

The cultures were set up in the greenhouse at 21°C without aeration, as aeration not only results in the production of root hairs, but also causes sugar depletion (Hoagland and Broyer, 1936). The water was changed on the 5th day to reduce the bacterial scum which formed on the surface of the water. The roots were excised on the 8th day; at this stage the roots were about 5 cm. long, without lateral root formation, and

with relatively few root hairs. The roots were cut off below the grain with a pair of scissors, placed in a beaker, and thoroughly washed in running tap water, or in distilled water, to remove any bacterial contaminants which would interfere in carbon dioxide fixation experiments.

Initially galvanised wire netting was used instead of the glass frames to support the cotton netting, but this appeared to give rise to substances toxic to root growth and development.

In later experiments, when the rate of germination and growth of the second batch of seed was slower, the seed was covered with enamel dishes during the first four days, and this accelerated both the rate of germination and root growth.

Satisfactory germination and growth were obtained, although in some instances there was a difference in the size of the seedlings between the two cultures set up. In the later experiments a recurrent infection of Rhizopus occurred, and the material from the infected areas was discarded.

B. Pretreatment.

The excised root material was divided according to the number of pretreatment conditions under investigation. After rinsing in the pretreatment solution the root material was

aerated for 24 hours in 500 ml. beakers containing approximately 350 ml. of the solution. Aeration was provided by a small Dymax blower through sintered glass aerators, or, when more than three forms of pretreatment were run, through drawn-out glass tubing. The beakers were supported on a wire gauze platform, held in a constant temperature bath at 25°C. Both initial and final pH measurements of the pretreatment media were made electrometrically.

A pretreatment procedure was adopted to allow the exposure to carbon-14 dioxide to be carried out under uniform conditions. Moreover, the use of phosphate buffer as an exposure medium enabled a constant reaction to be maintained during the exposure period (Pool, 1953).

At the end of the 24 hour pretreatment period the root material was washed with distilled water and blotted. Aliquots of 0.7 gm. fresh weight were taken for subsequent exposure to carbon-14 dioxide.

C. Preparation of labelled sodium bicarbonate.

In earlier experiments the isotope was supplied as sodium bicarbonate, but owing to the cost and the difficulty of obtaining bicarbonate supplies of sufficiently high specific activity, it was decided to convert barium carbonate, which is

much more readily available.

Labelled bicarbonate was prepared from the barium carbonate by the liberation of the carbon dioxide by acid, and absorption of the carbon dioxide in an excess of carbonate-free alkali hydroxide, the excess alkali being back-titrated with dilute acid (Calvin et al, 1949). The volumes of the reagents used were such that the supply of barium carbonate containing 1 millicurie of carbon-14 was converted to 4 ml. neutralized sodium bicarbonate, and thus the 200 µl. aliquots used in each exposure contained approximately 50 µc. activity.

Carbonate-free sodium hydroxide was prepared by slow solution of the metal in a vacuum desiccator (Calvin et al, 1949). A weighed sample of clean sodium in a glass container was placed in a vacuum desiccator over water. The desiccator was evacuated by a water pump. By the following morning, the sodium had completely dissolved, and the pressure in the vacuum desiccator was reduced with carbon dioxide-free air. The container with the dissolved sodium was carefully and quickly transferred to a flask containing carbon dioxide-free distilled water and protected by a soda lime guard tube. The flask was connected to a side-arm burette, also protected by a guard tube, and the sodium hydroxide was standardized against 0.01 N. hydrochloric acid, using methyl orange as an

indicator (Cumming and Kay, 1945).

The micro apparatus used in the wet combustion of root residues (see below) provided a suitable means for carrying out the barium carbonate conversion (Allen et al, 1947). The labelled barium carbonate was weighed in a small container, and carefully introduced into the reaction vessel. Conc. sulphuric acid (2.5 ml.) was run into the introducing vessel. The required volume of sodium hydroxide was delivered into the absorption vessel.

The apparatus was evacuated to 15 mm. Hg by a water pump. The concentrated sulphuric acid was slowly added to the barium carbonate sample by rotating the introducing vessel, and the reaction vessel was gently heated with a bunsen flame to drive the evolved carbon-14 dioxide over to the absorption vessel, which was immersed in an ice-water bath. The absorption period was 25 minutes with frequent intermittent shaking of the apparatus. At the end of the period the pressure within the apparatus was checked before letting it down with carbon dioxide-free air.

A mixed indicator (Simpson, 1924) was used as being specially suitable for bicarbonate-carbonate titrations. Two drops of this indicator were added to the absorption vessel. It is essential that in the titration the acid

must be capable of very fine delivery, particularly near the end-point. An Agla micrometer syringe was used for this purpose (Francis et al, 1954), and it was provided with a long glass jet dipping below the surface of the solution. The titration was carried out with continual shaking to ensure thorough mixing, until the titration mixture underwent a fairly sharp colour change from blue to rose-pink. The titration mixture containing the labelled sodium bicarbonate was stored in a refrigerator (-10°C) until required.

D. Exposure to carbon-14 dioxide.

Barcroft flasks were used as exposure vessels, the manometers being operated with the compensation stopcocks open throughout. The aliquot of root material was cut at random into short lengths, and these were introduced into the Barcroft flask, which contained 5 ml. phosphate buffer (pH 5.6 - prepared according to Umbreit et al, 1949). The manometers were assembled, fitted on the shaker, and equilibrated for 15 minutes (30 min. in Experiment Nos. 24, 25, 27, and 38) at 25°C. At the end of the equilibration period the reaction side stopcocks were closed, and the isotope was introduced in the form of labelled sodium bicarbonate, which liberated carbon-14 dioxide on contact with the phosphate buffer.

A constant volume of the labelled bicarbonate was used in each experiment containing approximately 50μc. carbon-14. Owing to the varying specific activities of the successive supplies of the isotope, the partial pressure of the carbon dioxide varied for each experiment. It was therefore necessary to run controls for each experiment, not only on account of the variation of the isotope supplies, but also owing to the metabolic differences between successive batches of root material.

In the early experiments (Nos. 24, 25) the isotope was introduced after the equilibration period by means of a syringe pipette, which necessitated opening up the manometers. This undesirable feature was later avoided by the use of Keilin tubes - small glass cups each provided with a hook so that they could hang on the rim of the centre well of the Barcroft flask. The isotope was syringe-pipetted into the Keilin tube when setting up the manometer. The Keilin tube was later dislodged by vertically shaking the manometer at the end of the equilibration period.

The root material was exposed to carbon-14 dioxide for 60 minutes with continuous shaking. Manometer readings were taken after 5 min. and 60 min. intervals, and the changes in pressure in the reaction vessels were recorded. At the end of exposure period, the manometers were opened in the fume

cupboard, the phosphate buffer was decanted off, and the roots rinsed with distilled water; it was necessary to remove the phosphate buffer, as it would interfere with the subsequent chromatographic separation (Balston and Talbot, 1952). Boiling 80% ethanol was then poured on to the roots.

E. Preparation of the 80% ethanol extract.

The ethanol in the manometer flask was filtered through a weighed Whatman No.1 filter paper (9.0 cm. diameter) into a 50 ml. stoppered measuring cylinder, while the roots were transferred to a mortar and thoroughly ground up. The suspension of ground root material was quantitatively transferred to the filter paper, and further boiling 80% ethanol was poured on. On cooling, and once the filter paper had completely dried, the extract was made up to the 50 ml. mark.

According to Woodward and Rabideau (1953) prolonged treatment is required for the complete extraction of the 80% ethanol-soluble radioactive products of corn shoots. The efficiency of the extraction was therefore checked by refluxing the root residue with 25ml. 80% ethanol for periods up to 8 hours. The ethanol was filtered off, and aliquots were plated and counted for radioactivity. Negligible activity was found.

Plates were prepared of the ethanol extract by discharging 200µl. aliquots from a syringe pipette on to numbered glass discs (microscope cover glasses - No.3, 3/4" diameter). The discs were lightly ground on the lower surface, as this enabled the plating of the extract, and its evaporation to be more easily observed.

The plate was mounted on a turntable apparatus similar to that of A. A. Benson (Calvin et al, 1949). As the aliquot was carefully run on to the revolving plate, it was slowly evaporated by a warm air stream from a bunson and blower assembly.

The plates were counted using a thin end-window G.E.C. type BHM2 Geiger-Müller tube connected to a Labgear scaling unit. Triplicate plates were prepared for each extract, and from these the activity of the total extract was calculated.

F. - Treatment of the 80% ethanol insoluble residue.

The ground, ethanol-insoluble root residue, collected on a tared filter paper, was weighed to give the amount of dried root residue for each exposure. Each root residue was transferred to a screw-top vial for storage.

Carbon-14 fixed in the ethanol-insoluble fraction was determined by oxidising aliquots of the root residue by a

"wet combustion" method. The procedure used initially was that described by Calvin et al (1949), but the results obtained were unsatisfactory, and a full investigation of the method was carried out (see Section 111).

The root residue sample was oxidised to carbon dioxide which was absorbed in baryta and the excess alkali was neutralised by hydrochloric acid. The barium carbonate suspension from the absorption vessel was centrifuged and the supernatant barium chloride solution was poured off. The barium carbonate slurry was shaken up with distilled water, and plated by a filtration method (Armstrong and Schubert, 1948) using a specially constructed small brass Buchner funnel. A weighed filter paper planchet was placed over the filter disc. The planchets were cut with a No.12 (1.8 cm. diameter) cork borer from Green's Hyduro 808 filter paper; this filter paper, similar to Munktell's No.OK filter paper recommended by Calvin et al (1949), was found to be more satisfactory than Whatman Nos. 5, 40, and 42 filter papers for holding the barium carbonate precipitate. The planchet was moistened with water and suction from a water pump applied. A brass sleeve, with an internal diameter of 1.23 cm. at its top rim, was screwed down over the planchet. The barium carbonate was spread evenly over the planchet under suction by means of a syringe

pipette. Once the planchet was covered with an even layer of barium carbonate, it was washed through twice with distilled water to remove any barium chloride present, and finally dried with acetone and ether. The brass sleeve was unscrewed, the suction turned off, and the planchet with the barium carbonate removed and allowed to stand for several hours at balance-room temperature before being weighed. Generally two plates were prepared of the barium carbonate from each combustion.

The plates were counted with the thin end-window Geiger-Müller tube as before, the plates being screwed down in a brass holder. The specific activity was determined after correcting for the self-absorption factor, using the values given by Calvin et al (1949). The limited amount ob barium carbonate available prevented the preparation of infinitely-thick samples. Fractionation in the combustion rate of the carbon-14 compounds (Beamer and Atchison, 1950) was not evident, as duplicate plates for each combustion in general gave uniform values for specific activity. Any plates showing an unevenness, particularly at the edge, or cracks in the barium carbonate were discarded.

The combustion apparatus and the plating apparatus were checked periodically for cross contamination; samples

of benzoic acid were oxidised, and the barium carbonate plated and assayed for radioactivity. No activity was found in any of these determinations.

G. Radiochromatography.

Paper partition chromatography, initially used by Consden et al (1944), provides a relatively simple method for the qualitative analysis of mixtures of compounds. Its application to radioactive tracer studies was first demonstrated by Fink et al (1947) in studies on iodine-131 metabolism in rat tissues. The exposure of X-ray films to chromatograms yields radioautographs showing dark spots corresponding to the labelled compounds. Various investigations on plant metabolism using radiochromatography have been made, including those of Pool (1952,1953) and Kursanov et al (1953) on carbon assimilation in roots.

1. Preparation of samples for chromatography.

After the quantitative radioactivity assay had been made, the 80% ethanol-soluble extract was concentrated to 1-2 ml. by vacuum distillation at a temperature below 30°C to avoid loss of compounds by volatilization. The concentrated extract was transferred to a small screw-top

vial, and stored in a refrigerator until required.

An aliquot of 200 µl. of the concentrate was taken by means of a syringe pipette and applied to a circle, 2 cm. in diameter and 5 cm. from one corner of the filter paper sheet (Benson et al, 1950; Comar, 1955). The sample was slowly dried by a stream of warm air.

In the qualitative analysis of the ethanol-insoluble products, root residue samples were hydrolysed in 2 ml. of 6 N hydrochloric acid in sealed-off glass tubes at 15 lbs. pressure for 3 hours. On removal from the autoclave, the tubes were cracked open, and placed in a vacuum desiccator over concentrated sulphuric acid in the presence of potassium hydroxide pellets. The desiccator was evacuated by a water pump, and the tubes were left for a period of 5-6 weeks, until all the hydrochloric acid had evaporated off. Otherwise, the presence of the inorganic acid would have interfered with the running of the chromatogram (Consden et al, 1944; Partridge, 1948; Stark et al, 1951). To each hydrolysate 2 ml. distilled water was added, and the hydrolysates were stored in a refrigerator. For chromatography, 200 µl. or 400 µl. aliquots were applied to the filter paper sheets as described above. Owing to the difficulties encountered in the quantitative radioactivity determinations of the

ethanol-insoluble fraction, hydrolysates of the root residues were prepared only in certain of the experiments.

2. Chromatography.

Two-dimensional chromatograms were run on sheets of Whatman No.1 filter paper (18.3/4" x 22.1/2"). The solvents used were those recommended by Benson et al (1950):-

a) Phenol saturated with water at 23°C; this was later modified to 4 parts phenol : 1 part water, which could be more rapidly prepared, and gave equally good separation on the chromatograms.

b) Butanol-propionic acid-water which was prepared from equal volumes of

i) 1246 ml. n-butanol mixed with 86 ml. distilled water.

ii) 620 ml. redistilled propionic acid diluted with 790 ml. distilled water.

c) Owing to the cost of propionic acid, a butanol-acetic acid-water mixture was tried as the second solvent (Partridge, 1948). This was prepared from 74 ml. n-butanol, 19 ml. acetic acid, and 50 ml. distilled water (Benson et al, 1950). However chromatograms run with butanol-acetic acid-water as the second solvent, did not yield as good an overall separation as with the butanol-propionic acid-water.

The papers were suspended from stainless steel troughs (24" long, 2" diameter), to which was added approximately 140 ml. solvent for two papers. The papers were held in position by a steel rod placed over the interlocking folded edges of the papers. Removeable stainless steel "tunnels" fitted over the edges of the trough to facilitate the handling of the wet papers. The troughs were supported in chromatographic boxes (30" x 12" x 31") provided with glass ends, and with the interior surface heavily coated with paraffin wax. The lid of the box was held in position by 4 clamps, and was fitted with a rubber gasket to give a vapour-tight seal. Initially, each box contained one trough, but subsequently the boxes were each fitted with two troughs, enabling four chromatograms to be run simultaneously. A separate box was used for each solvent, and the two boxes were placed in a thermostated cabinet (34" x 40" x 38"), which was maintained at 25°C.

The chromatograms were run in the phenol solvent first, across the width of the paper; the time taken for both solvents to traverse the paper was generally 12-16 hours. On removal from the phenol solvent, the chromatograms were placed in a drying oven, which was provided with a heating element along the base shielded by a piece of sheet iron,

and with an outlet at the top through which the fumes were drawn by a small extractor fan and led outside the building. The chromatograms were heated intermittently to 90°C until all the phenol vapour had disappeared. They were then ready for running in the second solvent.

In early chromatograms oblique solvent fronts were particularly evident in the butanol-propionic acid-water solvent. The following factors were considered:-

a) Incomplete removal of the phenol.

To determine whether traces of phenol left on the paper were causing the oblique fronts, the order of the solvents was reversed so that the chromatograms were first run in the butanol-propionic acid-water without previous treatment in phenol. Under these conditions, the solvent fronts in the butanol-propionic acid-water were extremely obliquo, thus indicating that some factor was operative other than residual phenol.

b) Equilibration.

The effect of an equilibration period was examined by hanging the filter paper sheets in the chromatography boxes for 24 hours before running the chromatograms. A dish containing the solvent

was placed in the bottom of each box, together with two dishes containing the stationary phase (water) to saturate the atmosphere within the boxes.

Chromatograms which had been equilibrated still possessed oblique fronts, so that equilibration did not appear to be the governing factor. Since equilibration of the papers is recommended (Hanes and Isherwood, 1949; McFarren, 1951), the 24 hour equilibration period was maintained in the routine preparation of chromatograms.

c) Temperature.

The constant-temperature cabinet was heated by means of four 60 watt lamps placed along the bottom, two on either side, in series with a mercury toluene thermoregulator and hot-wire vacuum switch. Blank papers were run for which the rear lamps were removed. Under these conditions the lag in the solvent front was found to correspond with the position of the front lamps. This indicated that the method of heating the cabinet was unsatisfactory, involving localized heating of the chromatography boxes, which in turn affected the movement of the solvents (Consden et al, 1944).

The lamps were discarded, and instead a heating element was installed together with a fan to keep the air within the cabinet in continuous motion. The temperature in the cabinet between the boxes was measured and was found to be 28°C at the front compared with 25°C at the back and along the sides of the boxes. This was reflected in the solvent front of the chromatograms which sloped up towards the front of the cabinet. By placing partitions at the side of the cabinet towards the heating element and along the back of the cabinet to act as baffles, it was possible to maintain the temperature throughout the cabinet at 25 ± 0.5°C. Under these conditions satisfactory chromatograms were obtained.

3. Radioautography.

Sheets of Ilford Industrial-G X-ray film were exposed to the chromatograms in X-ray exposure holders. The period of exposure was 2 weeks in the case of the 80% ethanol-soluble extracts, and 6 weeks for the acid hydrolysates. The radioautographs were developed in Ilford "blue label" ID-42 developer, generally for about 5 minutes, thoroughly washed in running water, and placed in Ilford IF-9 chrome-alum

fixing solution for 20 minutes. The radioautographs were washed for at least one hour before drying at room temperature.

4. Identification of compounds.

The general pattern of the labelled compounds (see Fig.1) was similar to that found by Pool (1952,1953); identification was therefore made by comparison with these earlier radioautographs, where the labelled compounds had been identified by running with standards.

Spraying the papers with ninhydrin, either a 1% solution in ethanol (Patton and Chism, 1951), or an 0.1% solution in n-butanol (Pratt and Auclair, 1948) located the amino acids present (Ruhemann, 1910). This confirmed that the range of labelled amino compounds formed were the same as had been found in the Berkeley experiments (Pool, 1952,1953). To obtain maximum development after spraying with ninhydrin, the papers were heated in the drying cabinet for 10-20 minutes (Pratt and Auclair, 1948; McFarren, 1951). As the ninhydrin colours tended to fade on storage the papers were sprayed with 1% cupric nitrate solution in ethanol which rendered the spots a permanent salmon-pink colour if stored under dry conditions (Keverau and Wieland, 1951; Levy and Chung, 1953).

Several labelled compounds remained unidentified; the spot (Unknown A.) formerly named 'tyrosine' (Poel, 1952,1953) was found after spraying with ninhydrin, not to be tyrosine, but to lie in very close proximity to the unlabelled tyrosine that is present. Attempts to identify Unknowns B, C, and D which were suspected to be fumaric, succinic, and glycollic acids were unsuccessful. The elution method described by Bassham (1949) was unsatisfactory when tested with small quantities of standard solutions of succinic and fumaric acids. Even after prolonged extraction the concentrated eluates failed to give a positive test to a pH indicator spray, whereas spraying the eluted filter paper strips showed the acids still to be present. Moreover, the pH indicator solution containing 400 mg. bromocresol green per litre 95% ethanol, as recommended by Lugg and Overell (1947,1948), for the location of carboxylic acids, did not appear to be sufficiently sensitive for the amounts of carboxylic acids present on the papers. Malic acid, present on the radioautographs as large dense spots, was only indicated as very small, often indefinite areas, on papers sprayed with the pH indicator solution. In no instance was there a visible yellow spot corresponding to Unknown B, C, or D. Standard solutions of malic, fumaric

and succinic were prepared, each containing 1 mg. per ml. Mixtures of equal volumes of malic and fumaric, malic and succinic, were made. Chromatograms were run of 100µl. samples of these mixtures, and also of malic, fumaric and succinic acids separately, and the papers were sprayed with bromocresol green solution. The positions of fumaric and succinic acids, in relation both to malic acid and to the solvent fronts, were found to correspond fairly well with those of Unknowns B and C.

The predominant reaction to the bromocresol green spray occurred in an unlabelled compound lying close to, or overlapping, aspartic acid. This compound also gave a marked molybdenum blue reaction when the papers were sprayed for the presence of sugar phosphates, indicating the presence of a large quantity of an unlabelled phosphorus acid. Its position corresponded to the labelled phosphopyruvic acid identified by Benson et al (1950).

Phosphate esters were detected using the spray-test solution described by Hanes and Isherwood (1949) and Balston and Talbot (1952), and that used by Benson et al (1950). In both cases the esters are hydrolysed and react to give molybdenum blue. The radioactive compounds occurring close to the origin gave the molybdenum blue

colour indicating the incorporation of the carbon-14 into the phosphoric esters of sugars.

The apparent absence of radioactivity in certain compounds as indicated by radioautography does not necessarily preclude the participation of these compounds in the carbon-14 metabolism. Differences in the required exposure time may account for apparent variations in the pattern of labelled compounds obtained. Jacobson (private communication) has suggested that this may account for his finding that glutamic acid and glutamine as the only labelled amino compounds in carbon-14 dioxide experiments with 'Sacramento' barley roots (Jacobson, 1955). Moreover, certain compounds may serve a catalytic function in the carbon dioxide metabolism and therefore would not form a "pool" of labelled material. Consequently they would not be readily detected by radiochromatographic technique.

The pattern of labelled fixation products was similar for all forms of pretreatment and therefore it was of interest to know the differences in relative activity occurring in response to the changes in root metabolism. At best, the densities of the spots on the radioautograph serve only as a rough indication of the relative carbon-14 activities in the various compounds. In the cases of

intensely active spots and very weak compounds, the radioautograph may be definitely misleading, the governing factor being the exposure period of the film to the chromatogram. It is therefore imperative to expose the film fully, and to use the radioautograph solely to locate the labelled compounds on the paper for counting. Such counting must be done before spraying the paper with ninhydrin, otherwise activity in the amino acids will be lost as carbon-14 dioxide (Ruhemann, 1911; Van Slyke et al, 1941).

5. Counting of the chromatograms.

A G.E.C. type 232 Geiger-Müller tube with a window diameter of 2" was used for this purpose. In most cases this window aperture was sufficiently large to include the whole area of the spot, although in certain cases, the malic acid spot had to be counted in sections. The exposed areas of the radioautograph were outlined on the chromatogram in pencil. The spot to be counted was carefully shielded to prevent interference from other radioactive compounds occurring in the same area. As the Geiger-Müller tube was unshielded, the background count tended to be high, and it was necessary to check it fairly frequently.

The Geiger-Müller tube was connected to an Ekco ratemeter. As the activity of some of the compounds was

low, relatively few pulses were recorded per second, and this led to difficulty in following the meter readings owing to oscillations, even using the highest time-constant. To obtain these readings, a pen-recorder was constructed in the Department. This consisted of a 5 mA. recording milliammeter movement, supplied by Evershed and Vignoles Ltd., with chart transport added. Deflections on the ratemeter dial were reproduced by the recorder on the chart paper, $3\frac{1}{2}$" wide, and transported at approximately 1" per 4 minutes. Satisfactory traces of the activities of the different compounds were obtained, from which it was possible to estimate the mean in each case. The relative activity of the various compounds was estimated as a percentage of the counts recorded for each chromatogram. Representative chromatograms from each experiment were counted.

III. Investigation of the ethanol insoluble activity.

An essential requirement for the reliable determination of the radioactive carbon present in the root residue remaining after extraction in 80% ethanol, is a combustion technique giving high and reproducible yields for total carbon. The wet oxidation method of Van Slyke and Folch (1940) is a convenient method for the routine combustion of samples for radioactivity assay (Kamen, 1948; Lindenbaum et al, 1948; Calvin et al, 1949; Claycomb and Hutchens, 1950; and Collins and Ropp, 1955).

A. Initial method.

1. Procedure.

Initially, the procedure followed was that described by Calvin et al (1949). Although no dimensions were given for the illustration of Barker's apparatus, the introduction of approximately 7 ml. oxidant fluid in a small vessel, necessitates a relatively large reaction vessel, and consequently the rest of the apparatus was made in proportion (see Fig. 2).

Van Slyke's combustion fluid, modified after Evans and Huston (1952), was used as the oxidant; their method

of preparation prevents the evolution of sulphur trioxide fumes during the combustion, and the oxidant fluid remains stable during a much longer period of storage. Hydrochloric acid (0.1 N.) was standardised against potassium iodate of known normality, in the presence of excess potassium iodide and sodium thiosulphate, using methyl red as indicator. Carbon dioxide-free barium hydroxide (0.2 N.) was prepared according to Cumming and Kay (1945) and was standardised against the hydrochloric acid using phenolphthalein as indicator.

The sample of root residue (2-5 mg.) was weighed on a capillary watch glass, and was introduced into the reaction vessel together with the small tube (the lower part of a 3/4" test-tube) containing approximately 5 ml. oxidant. The joints were greased with lanolin, and 5 ml. baryta was run into the receiving vessel from a burette. The apparatus was evacuated by a water pump and then shaken to empty the oxidant fluid onto the sample. The reaction vessel was gently heated for 15 minutes to drive off the carbon dioxide, then allowed to cool for 20 minutes, the apparatus being shaken intermittently throughout. At the end of the absorption period the vacuum within the apparatus was let down by opening the stopcock, the absorption vessel was removed, and

the excess barium hydroxide titrated with standard hydrochloric acid, using 0.5% phenolphthalein as indicator. From this, the volume of barium hydroxide converted to carbonate during the combustion was known, and therefore the percentage carbon yield for the sample could be determined. The yield could also be determined gravimetrically by transferring the barium carbonate precipitate to a weighed filter paper, and weighing the dried precipitate.

2. Results.

Determinations of the carbon yields were made for root residue samples from Experiments 24 and 25, and for benzoic acid, which serves as a standard of known carbon content (68.87%). The results given in Table 1. also include blank determinations, in which the sample was omitted.

Table 1/

Table 1.

Carbon yield determinations by wet oxidation - initial method.

Expt. No.	Flask No.	Treatment.	Sample mg.	% carbon yield. $(a)_x$	% carbon yield. $(b)_x$	specific activity. cts./min./mg. $BaCO_3$
24	2	Hoagland	5.48	28.1	-	-
"	"	"	2.94	41.4	-	23
"	"	"	2.37	44.1	27.7	-
24	6	Hoagland	4.88	36.2	-	-
"	"	"	2.51	40.1	-	22
"	"	"	1.77	31.1	26.8	-
24	8	Minus-K.	5.66	35.3	-	-
"	"	"	3.45	40.8	-	17
"	"	"	3.32	36.6	34.9	-
24	12	Minus-K.	4.20	38.9	-	26
"	"	"	5.40	29.2	-	-
"	"	"	3.68	43.5	-	34
"	"	"	1.49	61.3	-	-
25	2	Hoagland	4.72	40.7	-	21
"	"	"	2.46	30.9	32.6	-
25	6	Hoagland	4.75	35.2	-	11
"	"	"	3.65	34.9	-	-
"	"	"	4.06	30.0	32.9	-
25	10	Minus-N	4.83	44.9	-	20
"	"	"	5.85	37.0	-	26
"	"	"	3.61	31.6	34.3	-
25	12	Minus-N	2.58	50.8	-	-
"	"	"	3.00	49.4	-	29
"	"	"	2.39	42.3	42.7	-
Benzoic acid			4.38	66.9		
"			3.08	58.6		
"			1.86	48.0		
Blank				0.152 mg.	carbon	
"				0.171 "	"	
"				0.209 "	"	

x (a) - titrimetric determination.
(b) - gravimetric determination.

As the carbon yields for benzoic acid were proportional to the weight of the sample combusted, they indicated that the absorption of carbon dioxide was incomplete. In general, it was impossible to achieve a quantitative transfer of the barium carbonate from the absorption vessel (Weisburgher et al, 1952), however those gravimetric measurements which gave a higher carbon yield over that determined from the titration values, indicated that the titrimetric technique was unsatisfactory.

3. Modifications.

a) To improve the titrimetric determinations, the burette containing the baryta was changed to one which delivered through a stopcock instead of a rubber connection, and the hydrochloric acid was contained in a Class A burette.

b) A Geryk pump was used to evacuate the apparatus to a pressure lower than 15 mm. Hg.

c) Omission of potassium iodate from the oxidising fluid gives carbon yields that are 2-5% low (Calvin et al, 1949), owing to the loss of carbon as carbon monoxide. Therefore 0.3 gm. potassium iodate was added to the reaction vessel (Buchanan and Nakao, 1952).

d) The percentage carbon yields obtained in benzoic acid combustions, using these modifications, are given in Table 2.

Table 2.

Determinations of the carbon yield of benzoic acid (carbon content - 68.87%) by wet oxidation - initial method with modifications.

Sample mg.	% carbon yield.
3.78	60.8
3.66	38.0
2.74	58.4
3.02 x	41.5
3.91 x	50.7

x 0.3 gm. potassium iodate added.

It was evident that the method used failed to give quantitative transfer of the oxidised carbon from the sample; this has also been reported by Claycomb and Hutchens (1950). As the samples for combustion contained only 1-2 mg. carbon, it is probable that the apparatus was too large to obtain carbon analyses of these micro-samples.

B. Micro-Apparatus No.1.

1. Procedure.

An apparatus was designed to reduce the distance over which the carbon dioxide, liberated during the combustion, had to pass to reach the barium hydroxide solution. The dimensions of this apparatus (see Fig. 3) were comparable with those used by Lindenbaum et al (1948),

Thorn and Ping Shu (1951), Peters and Gutmann (1953), and Kornberg et al (1953).

Joints were greased with Dow Corning High Vacuum Silicone lubricant (Claycomb and Hutchens, 1950) to avoid possible organic contamination of the reaction vessel from the lanolin. The weighed sample was introduced into the reaction vessel in a small tube, together with 0.3 gm. potassium iodate. The oxidant was run into the introducing vessel through a small thistle funnel; during the combustion the oxidant could be added to the reaction vessel by rotating the introducing vessel through 180°. Evacuation of the apparatus was carried out by a water pump, as a pressure of 20 mm. Hg. had proved satisfactory to Lindenbaum et al (1948) and Peters and Gutmann (1953). At the end of the combustion period, the vacuum was let down with carbon dioxide-free air, by means of a 2-way stopcock, to prevent contamination with atmospheric carbon dioxide (Claycomb and Hutchens, 1950; Weisburgher et al, 1952; Kornberg et al, 1953).

The percentage carbon yields obtained in benzoic acid combustion are given in Table 3.

Table 3/

Table 3.

Determinations of the carbon yield of benzoic acid (carbon content - 68.87%) by wet oxidation, using micro-apparatus No.1.

Sample mg.	% carbon yield.
5.38	57.3
2.89	59.6
4.65	53.9

2. Modifications.

a) There was a tendency for air to leak in at the B 19. joint of the combustion apparatus, therefore all joints were carefully ground in. The water pump was turned on before assembling the absorption vessel, to provide a more rapid evacuation, and the final pressure at the completion of the absorption period was checked. The period of time for boiling was increased to 20 minutes, and the cooling period was increased to 45 minutes. The apparatus was thoroughly cleaned in chromic acid before each combustion.

The carbon yields for benzoic acid and glucose samples are given in Table 4.

Table 4/

Table 4.

Carbon analyses for Benzoic acid (carbon content - 68.87%) and Glucose (carbon content - 42.2%) using micro-apparatus No.1.

Material	Sample mg.	Final pressure. mm.Hg.	% carbon yield.
Benzoic acid	3.99	175	63.3
"	3.19	20	69.3
"	2.12	415	54.2
"	4.01	22	63.4
Glucose	4.32	30	40.7
"	4.21	410	38.5

These data confirmed that the maintenance of the pressure below 20-30 mm. Hg. throughout the combustion period is a critical factor (Claycomb and Hutchens, 1950; Thorn and Ping Shu (1951).

b) Since small variations in the volume of baryta used would result in large errors in the titrimetric determination of the carbon yields, a more accurate method of obtaining a reproducible volume of baryta was devised by using a constant volume delivery burette (see Fig. 4). This was provided with a wide jet to enable a rapid delivery of the baryta into the absorption vessel to be made. The volume of the baryta was determined both gravimetrically with distilled water, and titrimetrically against standard

acid. As the equivalent volume of standard acid was known, the percentage carbon yield could be expressed in terms of the standard acid used. An analysis of benzoic acid was carried out and yielded 63.4% carbon.

c) The oxidant was changed to that described by Van Slyke et al (1951), as this prevented the loss of carbon monoxide, due to the action of chromic anhydride in solution before the potassium iodate was dissolved by heating. It has the further advantage that both the liquid and solid reagents are stable, there is less tendency to interference from sulphur trioxide fumes on heating during the combustion, and 5 minutes boiling is sufficient for complete oxidation.

The solid reagent was introduced into the reaction vessel in a small tube, similarly to the sample, in order to prevent any adhering to the walls of the combustion vessel.

The results for two combustions of benzoic acid samples are given in Table. 5.

Table 5/

Table 5.

Carbon analysis for Benzoic acid (carbon content - 68.87%) using micro-apparatus No.1, and the oxidising reagents recommended by Van Slyke et al (1951).

Sample mg.	Final pressure mm. Hg.	% carbon yield.
5.91	37	65.1
3.88	21	63.2

d) The importance of temperature in relation to the complete absorption of the carbon dioxide has been emphasized by Claycomb and Hutchens (1950), and Peters and Gutmann (1953). The absorption vessel was held in an ice-water bath; this not only increased the efficiency of the absorption, but also the overall speed of the combustion.

The carbon yields for benzoic acid and dried root samples are given in Table 6.

Table 6.

Carbon analyses for Benzoic acid (carbon content - 68.87%) and dried root material using micro-apparatus No.1 with the absorption vessel immersed in an ice-water bath.

Material	Sample mg.	Final pressure mm. Hg.	% carbon yield.
Benzoic acid	3.74	16	65.9
"	4.22	31	94.1
"	3.57	20	66.2
Dried roots	3.57	15	38.5
"	3.74	15	39.5
"	3.10	15	40.4

C. Micro-Apparatus No.2.

1. Initial form of the apparatus.

This apparatus (see Fig. 5) was designed to eliminate the horizontal section connecting the oxidation and absorption vessels, and therefore to reduce the distance over which the carbon dioxide had to pass. It was also designed to facilitate both the introduction of the sample and the solid reagent by a vertical opening to the reaction vessel, and the titration of the excess baryta by having a vertical, rather than a curved, absorption vessel, provided with a wide opening.

The percentage carbon yields obtained for samples of benzoic acid are given in Table 7.

Table 7.

Carbon analyses for benzoic acid (carbon content - 68.87%) using micro-apparatus No.2.

Sample mg.	Final pressure mm. Hg.	% carbon yield.
3.53	15	71.7
4.81	25	58.9
4.64	65	56.6
2.58	15	59.9
4.56	23	59.3
1.86	15	57.6

This form of apparatus was found to be unsatisfactory in practice, as the baryta in the absorption vessel splashed up into the cone, on the addition of the liquid oxidant

reagent to the sample.

2. Modification of micro-apparatus No.2.

The B 19. socket and cone were interchanged, and the receiving vessel was restored to the curved form of micro-apparatus No.1. The position of the introducing vessel was moved below that of the side arm (see Fig. 8).

Table 8. gives the carbon yields obtained for benzoic acid and dried root material, and also includes the results for blank determinations.

Table 8.

Carbon yield determinations using the modified form of micro-apparatus No.2.

Material	Sample mg.	Final pressure mm. Hg.	% carbon yield.
Benzoic acid	3.08	15	65.1
"	4.64	55	65.5
"	4.24	15	68.7
"	5.27	15	64.7
"	5.27	15	66.6
"	5.73	15	67.4
"	3.69	15	67.9
Dried roots	3.93	15	39.5
"	2.74	15	41.8
"	2.48	15	44.5
"	4.48	15	43.7
Blank	-	15	0.081 mg. carbon
"	-	15	0.251 " "
"	-	15	0.183 " "

Two further sets of apparatus (Nos. 3 and 4) were made similar to the modified form of apparatus No.2.

The results for combustions of benzoic acid samples are given in Table 9.

Table 9.

Carbon analyses of benzoic acid (carbon content - 68.87%) using apparatus Nos. 2, 3, and 4.

Sample mg.	Apparatus No.	Final pressure mm. Hg.	% carbon yield
4.85	3	15	63.1
3.85	4	415	33.2
5.13	2	17	70.0

The low value for the percentage carbon yield using apparatus No.4, was due to the fall in vacuum caused by a small capillary hole in the absorption vessel.

Table 10 gives the carbon yields obtained for combustions of root residue samples.

Table 10.

Carbon analyses for root residue samples using apparatus Nos. 2 and 3.

Expt. No.	Flask No.	Treatment	Sample mg.	Apparatus No.	% carbon yield	Specific activity cts/min/mg $BaCO_3$ (mean)
24	2	Hoagland	3.49	3	43.1	53
"	8	Minus-K.	4.48	2	44.0	42
25	2	Hoagland	3.61	3	40.9	39
"	6	Hoagland	4.22	2	41.9	45
"	12	Minus-N.	3.91	3	43.0	43

3. To avoid variations in the titrations with hydrochloric acid, care was taken to use the same region of the burette in the standardisation of the hydrochloric acid, in the determination of the acid equivalent of the baryta from the fixed-volume burette and in the neutralization of the excess baryta from the combustion.

The results of carbon analyses for benzoic acid, blank determinations, and root residue samples, are given in Tables 11, 12 and 13 respectively. The figures for the analyses are not corrected for the blank carbon values.

Table 11.

Carbon analyses for Benzoic acid (carbon content - 68.87%) using apparatus Nos. 2, 3 and 4.

Sample mg.	Apparatus No.	% carbon yield
3.42	2	71.0
3.40	2	70.3
2.96	2	65.6
5.09	3	70.9
2.41	2	68.9
4.34	2	68.7
2.52	3	68.5
5.01	4	67.7
4.01	2	67.0
4.25	4	68.5
3.09	2	70.5
4.77	4	67.7
3.34	3	68.0
3.08	2	69.4

Table 12.

Carbon yields in blank determinations using apparatus Nos. 2, 3 and 4.

Apparatus No.	Carbon yield mg.
2	0.124
3	0.137
3	0.110
3	0.082
4	0.064
3	0.070
2	0.064
2	0.084
3	0.028
2	0.035
3	0.064

Table 13.

Carbon analyses for root residue samples using apparatus Nos. 2, 3 and 4.

Expt. No.	Flask No.	Treatment	Sample mg.	% carbon yield	Specific activity cts/min/mg $BaCO_3$ (mean)
24	2	Hoagland	2.82	45.8	53
"	8	Minus-K	3.90	41.0	38
25	2	Hoagland	3.18	44.5	38
"	2	Hoagland	3.24	44.1	40
"	6	Hoagland	3.47	44.7	43
"	6	Hoagland	2.90	48.1	40
"	10	Minus-N	1.95	40.1	40
"	12	Minus-N	3.77	43.3	50
"	12	Minus-N	3.38	45.8	46
"	12	Minus-N	2.76	45.3	40

The improvement in the method was evident not only in the percentage carbon yields, but also in the approximately 100% increase in the specific activity of the barium carbonate, formed in the combustion of root residue samples from Expt. 24, and 25, over the values obtained by the original procedure.

D. Determinations of the insoluble activity in root samples from Expt. 27.

A series of combustions were carried out on samples of root residues from Expt. 27, using the modified method described above. The results for the carbon analyses, and for determinations of the specific activity of the barium carbonate, are shown in Table 14.

Table 14.

Results for combustions of root residue samples from Expt. 27, using apparatus Nos. 2, 3 and 4.

Flask No.	Treatment	No. Combustions	% carbon yield. (mean)	Standard deviation	Specific activity cts/min/mg.$BaCO_3$ (mean)	Standard deviation.
2	Hoagland	5	40.4	3	49	16
4	Hoagland	3	41.6	1	72	10
6	Hoagland	5	45.0	0	87	12
8	Minus-P.	4	40.8	3	68	6
10	Minus-P.	3	42.2	1	76	5
12	Minus-P.	3	41.4	2	58	1
14	Tap water	5	42.1	3	71	6
16	Tap water	5	43.7	2	72	8

It is evident that the data for the specific activity of different samples of the same material were extremely variable. As the values for the blank determinations were low and fairly consistent, errors due to either atmospheric carbon dioxide, or sulphur trioxide fumes from the oxidant were unlikely. Since carbon yields for benzoic acid were good, it was concluded that the apparatus and the technique permitted satisfactory diffusion and absorption of the carbon dioxide evolved from the oxidised sample.

In one instance where excess acid had been added during the titration, it was noted that the resulting specific activity was markedly lower than in the other determinations. This suggested that the effect of a slight excess of hydrochloric acid might have a marked influence on the activity of the barium carbonate.

B. Investigation of the effect of excess hydrochloric acid on the titration of baryta in the presence of barium carbonate.

1. Manometric determinations.

Barcroft manometer flasks were set up containing the normal titration mixture obtained in the combustion method, which included barium hydroxide from the constant volume

burette, a known weight of barium carbonate, 2 drops of 0.5% phenolphthalein as indicator, and 0.1 N hydrochloric acid added almost to the phenolphthalein end-point.

Hydrochloric acid (0.1-0.2 ml.) was added to Keilin tubes by means of a syringe pipette, and these were hung on the centre wells of the manometer flasks. Control flasks were set up in which this additional acid was omitted.

After a short equilibration period the Keilin tubes were dislodged from the centre wells so that further hydrochloric acid came into contact with the titration mixture. Any changes in pressure were recorded.

It was found that in every case where the end-point was reached and the phenolphthalein became colourless, there was an increase in pressure in the reaction flask, indicating evolution of carbon dioxide. In neither the control flasks, nor in the cases where the end-point was not reached, was there a marked change in pressure. The results were taken to demonstrate the qualitative rather than quantitative changes, as the conditions of temperature, pressure, and volume of acid present were not rigidly controlled.

2. pH determinations.

a) pH determinations were made, using a Cambridge pH meter with a Morton cell, of titration mixtures which

were close to the phenolphthalein end-point.

i) The effect of excess 0.1 N and 0.01 N hydrochloric acid was investigated. pH readings were as follows:-

Initial pH	pH after the addition of 1 drop 0.1 N HCl.	pH after the addition of 1 drop 0.01 N HCl.
8.26	5.94	7.83
8.18	6.68	7.78

Therefore 1 drop excess 0.1 N hydrochloric acid had a marked effect in lowering the pH of the titration mixture.

ii) pH measurements were also made of a solution containing barium carbonate and barium chloride in the proportions generally encountered after a combustion, and also of the baryta and hydrochloric acid titration mixture with and without added barium carbonate. But, owing to the tendency for the pH values to drift, it was difficult to make exact comparisons of the readings.

iii) A similar difficulty was found in determining the pH of the titration mixture near the end-point of a mixed indicator (Simpson, 1924), nor did the readings agree with the theoretical values.

b) A series of electro-titrations were carried out using dip electrodes, as a more satisfactory method of

studying the changes in pH throughout the titration. Electro-titrations of baryta with added barium carbonate using i) 0.1 N, and 0.01 N hydrochloric acid with phenolphthalein as indicator, and ii) 0.1 N hydrochloric acid with Simpson's mixed indicator, were made. The results are shown graphically (see Fig. 7). Again difficulty was experienced in obtaining stable pH readings in the regions where the values were changing rapidly.

There did not appear to be any advantage in using the mixed indicator; it had a similar pH range as phenolphthalein, and its colour changes were less definite to follow.

It was evident from these investigations that 0.01 N hydrochloric acid was more suitable for titrating in the region of the end-point.

Literature regarding the titration of alkaline hydroxide in the presence of carbonate is divergent. Treadwell and Hall (1945) state that an alkaline hydroxide in the presence of insoluble carbonate may be titrated with the acid, and that the phenolphthalein end-point is obtained as soon as the hydroxide has been completely neutralized before any of the insoluble carbonate dissolves. Kuster (1897) found that

the use of phenolphthalein was unsuitable for the estimation of carbonate in free alkali, and McBain (1912) also states that the significance of the colour change varies enormously with the amount of carbonate present. Simpson (1924) observed that the gradual colour change in the phenolphthalein made the definition of the exact end-point difficult, although in the present investigation this was found easier to follow than the change for thymol blue, which is recommended by Christensen and Wong (1941) and Thorn and Ping Shu (1951).

Simpson (1924) also reported that the concentration of the phenolphthalein had a very considerable effect on the end-point, a factor also commented upon by Vorländer and Strube (1913), who found that the observed time for decolourization of the mixture was influenced by the amount of the phenolphthalein indicator present.

Several workers (Calvin et al, 1949; Van Slyke et al, 1951; Peters and Gutmann, 1953) state that the titration of excess baryta in a combustion may be carried to the phenolphthalein end-point. However Kuyper and Jones (1941) and Hutchens et al (1950) strongly recommend that the solution containing the barium carbonate should remain just

pink to phenolphthalein to prevent any loss of carbon dioxide, which is in agreement with the observations made here.

Moreover, the careful, slow addition of the acid to the titration mixture with constant shaking is essential, if local acidification of the solution is to be prevented (Friedman and Kendall, 1929; Mitchell, 1935; Kuyper and Jones, 1941; Gaboural et al, 1955).

F. Further determinations of the insoluble activity in root residue samples from Expt. 27.

Further combustions were carried out on samples of root residues from Expt. 27, in which the following precautions were taken in the titrations:- i) 0.1 N hydrochloric acid was added slowly with continual shaking of the vessel, ii) 0.01 N hydrochloric acid was used near the end-point, and iii) the titration was stopped just before the phenolphthalein end-point was reached, when the solution was still a faint pink.

Determinations of the percentage carbon yield from the titration values were complicated by the use of the two strengths of acid, and as interest was in the specific

activity measurements, routine determination of the percentage carbon yield was omitted.

The results for the insoluble activities are given in Table 15.

Table 15.

Insoluble activity determinations for root residue samples from Expt. 27, using apparatus Nos. 2, 3 and 4, and the modifications to the titration procedure described in the text.

Flask No.	Treatment	No. Combustions	No. Plates	Specific activity cts/min/mg $BaCO_3$ (mean)	Standard deviation.
8	Minus-P	5	10	61	9
10	Minus-P	5	10	64	2
14	Tap water	2	4	62	2

The effect of the addition of excess oxidant was checked and found to bear no relation to the results. It was observed that, in certain cases, the specific activities were influenced by the time taken for the titration, lower values occurring with increased time. This suggested the absorption of atmospheric carbon dioxide resulting in the formation of inactive barium carbonate. An alternative cause could have been due to variation in the carbon-14 content of the root material itself, particularly as a linear zonation of fixation was found to occur in the roots. To obviate this factor, the root residues were thoroughly reground in 80% ethanol.

G. Final modifications.

Two further modifications were investigated.

1. The absorption vessel of the combustion apparatus was modified so that the barium carbonate could be filtered directly from the absorption vessel under vacuum onto a sintered glass disc (No.4 porosity), and the excess baryta washed through with carbon dioxide-free distilled water. The barium carbonate was recovered on a small filter paper disc placed over the sintered glass disc (see Fig. 8).

In this way, any errors arising from the titration were avoided, and interference from atmospheric carbon dioxide was precluded. Removal of the excess baryta by filtration, rather than by neutralization, was the method used in the wet combustion procedures of Darben et al (1947), Lindenbaum et al (1948) and Evans and Huston (1952).

2. The titration of the excess baryta was maintained, but the absorption vessel was fitted with a vertical cone in addition to the side one. This facilitated the carrying out of the titration and enabled a rubber cap, through which the burette jet passed, to be fitted over the vertical cone (Christensen and Wong, 1949; Hutchens et al, 1950), while the side cone, after being disconnected from the reaction vessel, was quickly stoppered with a sealed-off socket.

The titration could be carried out slowly and with constant shaking, free from interference by atmospheric carbon dioxide.

3. Results.

a) Recovery of barium carbonate.

To determine the percentage recovery of barium carbonate, which would be obtained in the normal combustion procedure, weighed samples of benzoic acid were oxidised and the barium carbonate formed was plated as in the routine determination of the insoluble activity. The weight of the barium carbonate recovered was expressed as a percentage of the calculated weight of barium carbonate formed from the benzoic acid, assuming a 100% oxidation and absorption of the benzoic acid. The results are given in Table 16.

Table 16.

Recovery of barium carbonate by the filtration and modified titration techniques, expressed as a % of the calculated weight of the barium carbonate formed in the oxidation of the benzoic acid samples.

Method	No. determinations	% $BaCO_3$ recovery (mean)	Standard deviation
Filtration	6	65.2	3.7
Titration	2	83.1	6.8

The recovery of barium carbonate was consistently lower by

the filtration technique, a considerable quantity of the carbonate being left adhering to the walls of the absorption vessel. More important, too, was that the barium carbonate recovered by this method was found to be unsatisfactory for plating; the carbonate collected on the filter paper disc under vacuum, did not readily go back into suspension, even in the presence of added alcohol or detergents to reduce surface tension. Only by centrifuging, followed by grinding with a glass rod, was it possible to obtain barium carbonate which gave smooth plates.

b) Specific activity determinations.

Results for specific activity determinations of the barium carbonate formed from the oxidation of root residue samples by the two methods are given in Table 17.

Table 17.

Specific activity determinations of barium carbonate from root residue samples by the filtration and modified titration techniques.

Expt. No.	Flask No.	Method	No. determinations.	Specific activity cts/min/mg $BaCO_3$ (mean)	Standard deviation
28	8,10,30	Filtration	3	9	0
28	8,10,30	Titration	6	9	1
29	13,15	Filtration	4	187	11
29	13,15	Titration	8	193	7

The results for both methods were in fair agreement. However the titration method was adopted in preference to the filtration technique, as it was more rapid, gave a higher recovery of barium carbonate, and the barium carbonate yielded good plates.

A comparison of the results with those obtained using the original titration method without the rubber cap is shown in Table 18.

Table 18.

Comparison of the specific activity determinations of barium carbonate from root residue samples by the modified technique with those of the original titration method without the rubber cap.

Expt. No.	Flask No.	Method	No. determinations	Specific activity cts/min/mg $BaCO_3$ (mean)	Standard deviation	
27	14	Original	2	62	2	x
27	14	Modified	3	103	3	
29	13,15	Original	2	183	3	
29	13,15	Modified	8	193	7	

x - see Table 15.

H. Determinations of the insoluble activities using the modified titration method.

The results for specific activity determinations of the barium carbonate from root residue samples are given in Table 19.

Table 19.

Specific activity determinations of the barium carbonate from root residue samples of 1) Expt. 29, 2) Expt. 31, 3) Expt. 35, and 4) Expt. 36, using the modified titration technique.

	Expt. No.	Flask No.	Treatment	No. combustions	Specific activity cts/min/mg $BaCO_3$ (mean)	Standard deviation
1.	29	1,3	Hoagland	8	143	5
	29	7,9	Hoagland + 1% glucose	7	153	8
	29	13,15	Distilled water	8	193	7
2.	31	2	Hoagland	2	61	1
	31	4	Hoagland + 0.001 M KCN	2	46	3
	31	8	Distilled water	3	63	5
	31	10	0.001 M KCN	2	61	2
	31	10	0.001 M KCN	2	486	9
3.	35	2	Distilled water	2	132	11
	35	4	Distilled water	2	110	1
	35	6	0.005 M KBr	2	133	6
	35	8	0.005 M KBr	2	120	10
	35	10	0.005 M $KHCO_3$	2	71	2
	35	30	0.005 M $KHCO_3$	2	75	6
	35	14	0.005 M $CaBr_2$	2	122	13
	35	16	0.005 M $CaBr_2$	2	117	9
4.	36	2	Distilled water	2	123	1
	36	4	Distilled water	2	90	11
	36	6	0.005 M KBr	2	124	5
	36	8	0.005 M KBr	2	116	4
	36	10	0.005 M $KHCO_3$	2	114	8
	36	30	0.005 M $KHCO_3$	2	156	2
	36	14	0.005 M $CaBr_2$	2	142	13
	36	16	0.005 M $CaBr_2$	2	178	6

I. Discussion.

The final results of the investigation were disappointing in that there was still a marked variation in the specific activity determinations.

Certain features remained unexplained, in particular two consecutive specific activity determinations when exceptionally high values were obtained (see Table 19, Expt. 31, flask 10). A possible explanation was sought in that the weighed samples had been present in the fume cupboard during the manipulations involved in the conversion of the labelled barium carbonate supply to sodium bicarbonate, and during the exposure period of another experiment. However, subsequent samples subjected to similar conditions, failed to yield high values for their specific activity.

The major disadvantage in the procedure is the micro-size of the samples available for combustion, owing to the limited amount of the root residues, and that a certain portion is required for hydrolysis. This factor renders the results liable to be influenced by variations in the blank values. Another serious source of error is the tendency for filter paper fibres to adhere to the root residue; even moistening the filter paper with 80% ethanol had little effect on reducing this. It is suggested that careful examination

of the root residues under a dissecting binocular, or preferably filtering off the 80% ethanol extract through a sintered glass filter, would be advantageous in rendering the root residues free from paper fibres.

As material for "wet combustion" is generally very limited, a method of direct plating of the root residue for determining the insoluble activity would be preferable. This would obviate the errors arising from the "wet oxidation" technique, and furthermore, the plated residues would be available for subsequent hydrolysis. An attempt to plate the ground residue was made, but even with thorough manual grinding, minute fibre fragments remain, and these lead to unevenness in the plates.

Although the trend of the insoluble activity determinations appeared to follow that of the soluble radioactivity assays, no reliability can be placed on the results in view of the numerous sources of error found in the method. Consequently, the ethanol-soluble activity was taken as the basis for comparison of the effects of different pretreatments.

IV. Zonation of carbon dioxide fixation.

Observations on the zonation of metabolic activity in roots have been made by several workers, Machlis (1944b) and Willis and Yemm (1955) investigated the respiratory gradient, but their results differ as to the region of maximum respiratory activity. The results of investigations on the occurrence of a linear zonation in salt accumulation are also at variance. Prevot and Steward (1936) and Steward et al (1942) found maximum salt accumulation towards the root apex. However Kramer and Wiebe (1952) observed a considerable variation in the distribution of phosphorus-32, which was also evident in the absorption of caesium-137 by single roots (Steward, 1954).

Wiebe and Kramer (1954) studied the translocation of various radioactive isotopes from different regions of the root, and observed a downward translocation and accumulation of the salts towards the apex. This is in agreement with the autoradiographic investigations of Rabideau and Moride (1953), who studied the translocation of labelled photosynthetic products to the roots and found that the greatest amount of radioactivity occurred in the root apex.

In order to investigate whether or not the magnitude of carbon dioxide fixation varies in different regions of the root, intact barley roots were exposed for 1 hour to carbon-14 dioxide after 24 hours pretreatment in aerated distilled water. The exposure period completed, the roots were arranged on a paraffin wax block, and by means of a multiple razor cutter, were divided into 3 batches of 17 mm. segments starting at the root tips. Each group of segments was treated separately with boiling 80% ethanol. Results for the total ethanol-soluble radioactivity and the relative activity of the different ethanol-soluble compounds are given in Tables 20 and 21.

Table 20.

Zonation of carbon-14 dioxide fixation by excised roots of barley - radioactivity of the 80% ethanol soluble extract.

Intact roots 0.7 gm. wet wt. 24 hrs. pretreatment in aerated distilled water. Exposure medium: 5 ml. PO_4 buffer (pH 5.6), 200 µl. $NaHC^{14}O_3$ containing approx. 50 µc. C^{14} and 17 µmol. CO_2. Time of exposure 1 hr. Roots cut into 3 batches 17 mm. segments from the tips.

Region. mm. from apex.	Radioactivity of 80% ethanol extract. cts/min.	Mean	Standard deviation	Mean as % 0-11 mm. sgt.
0-17	14,000 14,000 14,000	14,000	0	
17-34	9,500 9,000 8,000	8,833	624	63.1
34-51	6,000 6,500 6,500	6,333	236	45.2

The use of the t test showed that (at P=0.001) the radioactivity in the 0-17 mm. segments to be significantly greater than 17-34 mm. segments, and similarly (at P=0.01) that of the 17-34 mm. segments to be significantly greater than 34-51 mm. segments.

Table 21.

Zonation of carbon dioxide fixation by excised barley roots - relative activity of the labelled ethanol soluble compounds, obtained by counting chromatograms.

Region. mm. from apex. :-	0-17	17-34	34-51
Compound.	% activity counted.		
Malic	82.1	79.4	79.8
Citric	-	-	-
Aspartic	1.0	4.6	1.1
Glutamic	4.2	6.1	7.4
Serine	2.1	1.5	2.1
Asparagine	2.8	0	1.1
Glutamine	2.8	3.0	4.2
Alanine	-	-	-
PO_4 esters	-	-	-
Unknown A.	3.5	3.8	4.2
Unknown B.	1.4	1.5	-

- presence of compound not indicated on the radioautograph.

The results show that a marked zonation of carbon dioxide fixation occurs within the root, and that the fixation is greatest in the region of the root apex. The distribution of the carbon-14 in the labelled ethanol soluble compounds appears to be very similar in all the segments, with a very high proportion occurring in malic acid. Owing to the small amount of activity present on the chromatograms, it was not possible to detect the full range of labelled compounds, as some failed to give spots on the radioautographs during the exposure period of 2 weeks.

Whether or not the increased carbon dioxide fixation in the apical segments of the excised barley roots may be associated with a zonation of salt accumulation is open to question. Prevot and Steward (1936) found that the initial measurements after a 5 hour absorption period in low-salt barley roots did not show as marked a zonation for the different segments, as after 10 hours, or as in the high-salt roots. Thus it may be that a zonation of ion absorption would not be evident in low-salt barley roots during 1 hour exposure to 0.134 M phosphate buffer solution. If such a zonation does in fact occur, then the increased carbon dioxide fixation may be correlated with organic acid formation to balance excess cation absorption occurring in the tip.

Alternatively the results of Lundegårdh (1949b) may be relevant in accounting for the observed zonation of fixation. Lundegårdh demonstrated a metabolic differentiation between the 0-30 mm. and 30-60 mm. zones in wheat roots. Although both zones had an equal power of ion absorption, it was found that the distilled water respiration was twice as high in the tip zone, and that a "third respiration", sensitive to 0.001 N hydrocyanic acid, was present only in the tip zone. Distilled water respiration is interpreted as anion respiration by means of native anions, representing internally transported salts and organic acids, particularly the latter. The nature of the "third respiration" is open to discussion, but Lundegårdh suggests that it interacts with the organic acid systems which predominate in the tip. Therefore, the differentiation found in the wheat roots may be related to the zonation in the organic acid activity.

If a similar differentiation occurs in barley roots, it would lead to a greater decrease in the organic acid content in the region of the root tip during the distilled water pretreatment, and consequently to a greater production of organic acids, incorporating carbon-14 dioxide, in order to balance the excess cation absorption occurring in the presence of phosphate buffer, as suggested by Jacobson (1955).

The results of the experiment were of importance in connection with the quantitative determination of the radioactivity in the root residues by the "wet oxidation" method. The fact that carbon dioxide fixation is not uniform within the root, indicates that variation may occur in the samples for combustion, unless care is taken to grind and mix thoroughly the root residues.

As this zonation of fixation was not proved until after a number of the experiments on carbon dioxide fixation in relation to root metabolism had been carried out, the use of whole roots as samples for exposure to carbon-14 dioxide was maintained throughout the investigation. This probably accounted for the considerable variation found in the radioactivity of the 80% ethanol soluble products for experiments with the same pretreatment.

V. Carbon dioxide fixation and mineral ion absorption.

A. Pretreatment in Hoagland solution minus Potassium, Nitrogen, or Phosphorus.

The effect of pretreatment in a mineral solution on the subsequent carbon dioxide fixation (Pool, 1952, 1953) was further investigated to determine whether or not the observed decrease was due to a specific element. The mineral elements investigated were potassium, nitrogen, and phosphorus, as these were considered to play a major role in plant metabolism.

The complete mineral solution was that of Hoagland and Snyder (1933), modified in that ferric tartrate was omitted, as this tends to precipitate on the roots (Pool, 1953). Substitution of the mineral elements under investigation in the Hoagland formula was according to Table 22.

Table 22.

Compositions of the solutions used in the pretreatment of excised barley roots.

Solution	$Ca(NO_3)_2 \cdot 4H_2O$	$MgSO_4 \cdot 7H_2O$	KH_2PO_4	KNO_3	NaH_2PO_4	$NaNO_3$	$CaSO_4$	KCl
Complete	1.18	0.49	0.14	0.51	-	-	-	-
Minus K.	1.18	0.49	-	-	0.14	0.43	-	-
Minus N.	-	0.49	0.14	-	-	-	0.68	0.38
Minus P.	1.18	0.49	-	0.51	-	-	-	0.07

Root material was aerated for 24 hours in the mineral element deficient solution, and in complete Hoagland solution as control, and was subsequently exposed to carbon-14 dioxide for 1 hour. For each element, experiment and control were carried out in triplicate. In the minus-phosphorus experiment fixation after pretreatment in tap water was also measured. The radioactivity in the 80% ethanol soluble fractions are given in Tables 23, 24, and 25, and the distribution of the carbon-14 in the ethanol soluble compounds are given in Table 26.

Table 23.

The effect of pretreatment for 24 hours in Hoagland solution minus potassium on carbon-14 dioxide fixation by excised barley roots- radioactivity of the 80% ethanol soluble extracts.

Roots 0.7 gm. wet wt. Exposure medium:- 5 ml. PO_4 buffer (pH 5.6), 200 µl. $NaHO^{14}O_3$ containing approx. 50^4 µc. C^{14}, and 17 µmol. CO_2. Time of exposure 1 hour.

Pretreatment medium	Flask No.	Activity cts/min.	x Mean for Expt.	s.d.	xx	Mean for treatment	s.d.	xx	Mean as % of control
Complete	24/2	72,500 74,000 74,500 72,500	73,375	893					
Complete	24/4	65,500 69,000 68,500	67,667	1,546		70,403	2,336		
Complete	24/6	70,500 70,000 70,000	70,167	235					
Minus-K	24/8	43,500 43,500 42,000	43,000	707					
Minus-K	24/10	49,500 52,000 50,000 50,000	50,500	935		49,723	4,899		70.62
Minus-K	24/12	55,500 57,000 54,000	55,500	1,225					

x - radioactivity of the 80% ethanol extract obtained from plate (assays.
xx - standard deviation.

The t test was applied to the results both for experiments of the treatment, and also to the difference between the two forms of treatment.

		Level of probability.
a) Within the treatment.		
No.24/2 : No.24/4 -	significant	0.01
No.24/2 : No.24/6 -	"	0.01
No.24/4 : No.24/6 -	not significant	0.1
No.24/8 : No.24/10 -	significant	0.001
No.24/8 : No.24/12 -	"	0.001
No.24/10: No.24/12 -	"	0.01
b) Between the treatments		
Complete : minus-K -	significant	0.01

Table 24.

The effect of pretreatment for 24 hours in Hoagland solution minus nitrogen on carbon-14 dioxide fixation by excised barley roots - radioactivity of the 80% ethanol soluble extracts.

Roots 0.7 gm. wet wt. Exposure medium:- 5 ml. PO_4 buffer (pH 5.6), 200 µl. $NaHC^{14}O_3$ containing approx. 50 µc. C^{14}, and 17 µmol. CO_2. Time of exposure 1 hour.

Pretreatment medium.	Flask No.	Activity cts/min.	Mean for Expt.	s.d.	Mean for treatment	s.d.	Mean as % of control.
Complete	25/2	62,000					
		64,000	63,333	943			
		64,000					
Complete	25/4	56,500					
		59,000	58,333	1,312	59,111	3,176	
		59,500					
Complete	25/6	55,000					
		57,000	55,667	943			
		55,000					
Minus-N	25/8	75,000					
		77,500	76,333	1,028			
		76,500					
Minus-N	25/10	87,500					
		78,000	80,375	3,962	81,180	4,323	137.33
		77,000					
		79,000					
Minus-N	25/12	87,000					
		88,500	86,833	1,434			
		85,000					

	Level of probability
a) Within the treatment.	
No.25/2 : No.25/4 - significant	0.02
No.25/2 : No.25/6 - "	0.01
No.25/4 : No.25/6 - not significant	0.1
No.25/8 : No.25/10 - " "	0.3
No.25/8 : No.25/12 - significant	0.01
No.25/10: No.25/12 - not significant	0.3
b) Between the treatments	
Complete : Minus-N - significant	0.01

Table 25.

The effect of pretreatment for 24 hours in a) Hoagland solution minus phosphorus, and b) Tap water on carbon-14 dioxide fixation by excised barley roots - radioactivity of the 80% ethanol soluble extracts.

Roots 0.7 gm. wet wt. Exposure medium:- 5 ml. PO_4 buffer (pH 5.6) 200 µl. $NaHC^{14}O_3$ containing approx. 50 µc. C^{14}, and 6 µmol. CO_2. Time of exposure 1 hour.

Pretreatment medium.	Flask No.	Activity cts/min.	Mean for Expt.	s.d.	Mean for treatment	s.d.	Mean as % of control.
Complete	27/2	107,500					
		110,500	110,333	2,248			
		113,000					
Complete	27/4	98,000					
		98,000	100,000	2,829	104,167	4,448	
		104,000					
Complete	27/6	108,500					
		97,500	102,167	4,643			
		100,500					
Minus-P	27/8	104,000	100,500	3,500			
		97,000					
Minus-P	27/10	102,000					
		99,500	100,500	1,080	100,111	550	96.11
		100,000					
Minus-P	27/12	100,000					
		100,500	99,333	1,312			
		97,500					
Tap water	27/14	117,500					
		116,500	117,167	472			
		117,500					
					119,167	2,000	114.44
Tap water	27/16	121,000					
		118,000	121,167	2,657			
		124,500					

	Level of probability
a) Within the treatment.	
No.27/2 : No.27/4 - significant	0.02
No.27/2 : No.27/6 - not significant	0.1
No.27/4 : No.27/6 - " "	0.7
No.27/8 : No.27/10 - " "	1.0
No.27/8 : No.27/12 - " "	0.8
No.27/10: No.27/12 - " "	0.4
No.27/14: No.27/16 - " "	0.1
b) Between treatments.	
Complete : minus-P - not significant	0.3
Complete : tap water - significant	0.05

Table 26.

The effects of pretreatment for 24 hours in Hoagland solution minus a) potassium, b) nitrogen, c) phosphorus, and in tap water on carbon-14 dioxide fixation by excised barley roots - relative activity of the labelled ethanol soluble compounds, obtained by counting chromatograms.

Treatment:-	Control	Minus-K	Control	Minus-N	Control	Minus-P	Tap water
Flask No.	24/2	24/12	25/4	25/10	27/2	27/10	27/14
Compound.	% activity counted.						
Malic	25.7	29.8	30.3	48.7	19.3	17.3	25.7
Citric	9.3	11.0	7.5	3.4	8.6	9.9	4.9
Aspartic	23.4	12.9	18.7	8.7	16.5	10.6	5.8
Glutamic	19.7	14.4	20.4	8.7	21.0	12.6	12.9
Serine	4.4	5.1	4.0	2.0	7.1	8.3	5.3
Asparagine	3.4	4.5	6.9	9.5	8.6	18.4	17.6
Glutamine	4.7	5.0	2.7	10.7	6.4	11.4	14.8
Alanine	0.8	0.9	0.6	0.7	0.6	0.9	1.2
PO_4 esters	1.0	1.8	0.1	0.9	0.9	1.9	2.0
α -ketoglutaric	0.3	1.0	-	-	-	-	-
Unknown A.	1.3	4.8	1.6	6.0	3.9	4.3	7.2
Unknown B.	0.9	0.4	0.5	0.1	0.8	0.4	-
Unknown C.	1.3	2.1	1.1	0.4	1.3	2.0	1.0
Unknown D.	-	0.4	-	-	-	-	-
Unknown E.	-	-	-	-	0.1	-	0.3
Unknown F.	1.7	1.2	1.9	-	1.7	1.6	0.1
Unknown G.	2.0	4.6	3.5	-	3.1	0.3	1.3

- presence of compound not indicated on the radioautograph.

A statistical analysis of the results in mineral ion and carbon dioxide fixation studies has generally been omitted in most of the published work. In the present series of experiments the t test was applied to determine the significance of the results obtained. Referring to Tables 23, 24, and 25, the values for t within a treatment show that a significant difference may occur between experiments with the same pretreatment. The probable source of variation is in the method of sampling the root material. The weighing out of 0.7 gm. aliquots of blotted roots, provides a rapid and easy method of sampling. Determinations were made of the weight of insoluble root residue recovered in each experiment, and considerable variation was found. These figures were subject to error in that the weight of root residue was very small in comparison to the weight of the filter paper on which it was collected. Moreover, the dry weights are not proportional to the fresh weights of the samples, unless the samples possess a uniform water content. The figures for the dry weight of the insoluble root residues did not correlate with those for the ethanol soluble radioactivity. Also, as linear zonation of carbon dioxide fixation has now been demonstrated, it is obvious that unless a known number of uniform roots or uniform segments is taken for all the samples, variations in

the carbon-14 dioxide uptake will occur. No such precaution was observed in the present investigation. The time necessary to count samples of a known number of segments, equivalent to 0.7 gm. was measured, and would have increased very considerably the time taken to set up the manometer flasks. It was considered that this time-lag would affect the metabolism of the roots, and might modify the conditions imposed by the form of pretreatment. In further work it would be advantageous to use only the root tips, and to weigh out the aliquots prior to pretreatment, thus avoiding variation due to zonation of metabolic activity, and reducing the time-lag at present involved between the pretreatment and exposure periods.

The t test was also applied to determine the significance of the difference in the carbon-14 dioxide fixation resulting from different forms of pretreatment. Although significant variations may occur between samples exposed to a single pretreatment, comparison of the results of different pretreatments is justified, in that the tendency for variation in the root samples is common to all the experiments. A significant difference between the results of experiments with one pretreatment will result in a high standard deviation for the mean value of that pretreatment, particularly as the

largest number of experiments for any one pretreatment was only 3. This high standard deviation for the mean value of a pretreatment will, in turn, lead to a low value of *t* when the results of two pretreatments are compared. This may considerably reduce the significance of the differences between different treatments or even prevent its detection (see probability levels for distilled water: 0.005 M $CaBr_2$ in Expts. 36 and 35).

This discussion of the *t* test analysis may be applied to all the experiments, and therefore it will not be restated in further experiments.

The results for the mineral-deficiency experiments show that pretreatment in a potassium-free solution results in decreased carbon-14 dioxide fixation compared with complete Hoagland solution, whereas with nitrogen omitted an increase of approximately similar magnitude is found. When phosphorus was omitted, fixation is slightly less than that for the control. The results indicate that for the mineral elements investigated, the effect of pretreatment in a nutrient solution on carbon dioxide fixation is not due to the presence of any one specific element.

The values for pretreatment in tap water versus Hoagland solution are of interest in that the marked increase in

carbon-14 dioxide fixation of tap water over mineral solution pretreatment reported by Poel (1952, 1953) was not evident.

Jacobson (1955) has suggested that the differences in the carbon dioxide fixation reported by Poel (1952,1953) are probably related to the extent of the excess cation absorption. Other investigators have shown that the excess cation absorption in roots leads to an increased organic acid content, particularly in malic acid (Ulrich, 1941; Overstreet et al, 1942; Lundegårdh, 1954; Jacobson and Ordin, 1954). Therefore, if carbon dioxide fixation is directly related to the organic acid requirements, considerable fixation should occur during excess cation absorption. As exposure to the carbon-14 dioxide takes place in the presence of phosphate buffer, the amount of potassium absorbed by the roots from this medium will be conditioned by their previous mineral ion status.

Substitution of potassium by sodium should have resulted in a lower cation uptake during pretreatment, and therefore to a greater ability to absorb potassium during the exposure period. Similarly increased anion absorption during pretreatment due to the substitution of phosphate by nitrate and chloride ions should have led to a greater potential for cation accumulation from the phosphate buffer. In the minus-nitrogen experiment, nitrate ions were replaced by chloride

and the less readily absorbed sulphate anions; the lowered anion uptake during pretreatment should have resulted in a decreased cation absorption during the exposure period, and therefore to a reduction in the carbon dioxide fixation.

The fact that the results obtained are not consistent with this hypothesis, suggests that the hypothesis is either incorrect, or that it is masked by secondary factors; these may arise from using a mixture of different salts, or that the metabolic status, such as the carbohydrate content, may influence the carbon dioxide fixation to a certain extent. The disadvantage of using a mixture of different salts, such as Hoagland solution, is that the relative uptake of ions is difficult to ascertain. Moreover, the effect on the relative anion:cation of substituting different salts may not necessarily be the same as would be obtained from these salts in single solution. Not only is there the factor of antagonism to be considered, but also the competition between different anions and different cations for "binding sites" (Jacobson et al, 1950; Overstreet et al, 1952; Epstein and Hagen, 1952; Epstein, 1953).

If the sugar content of the root determines in part the assimilation of carbon dioxide, then it is pertinent to consider the effect of deficiencies in the carbohydrate

metabolism. Potassium deficiency results in a marked lowering in the efficiency of carbohydrate synthesis (Russell, 1927), and a reduction in the sugar content (Gregory and Baptiste, 1936) due to the increased respiratory rate, which occurs under conditions of potassium deficiency (Pirson, 1955). On the other hand, under conditions of phosphorus deficiency, Gregory and Baptiste (1936) found a slight increase in the total sugar, which was also observed by Kraybill (1930), and is probably due to the reduction in protein synthesis (Richards and Templeman, 1936; Steinberg, 1951). The very marked reduction in protein synthesis under conditions of nitrogen deficiency (Kraybill, 1936; Richards and Templeman, 1936; Gregory and Baptiste, 1936) results in a large increase in the carbohydrate content (Kraybill, 1936; Gregory and Baptiste, 1936).

The distribution of the carbon-14 is of interest in relation to the metabolic effects of the deficiencies. The minus-potassium and the minus-phosphorus experiments in the present investigation differ from the minus-nitrogen experiment, in that the deficient elements are supplied during the exposure period. With potassium omitted, there is an increased fixation over the control in the malic and citric fractions. This could be indicative of an increased synthesis of sugars

to counterbalance their reduction which occurred during the pretreatment period. In the phosphorus-deficient experiment the amide fraction contains a higher proportion of the carbon-14. This may be related to the lowered protein formation relative to carbohydrate synthesis during the pretreatment period. In the case of minus-nitrogen the nitrogen-deficient conditions are still operative, and there is a marked increase in the percentage of the total activity incorporated into the malic acid, which correlates with the expected increase in sugar synthesis. As regards the nitrogenous compounds, an increase in fixation into the amide over the amino acid fraction is indicated. Willis (1951) found that amides, and in particular glutamine, were the primary synthetic products in excised barley roots following nitrogen deficiency.

Investigations on the effects of mineral deficiencies have yielded very divergent results (Steinberg, 1951), and very little work has been done on their effects on root metabolism. Therefore, until some facts are known about the actual effects of these deficiencies on barley roots, and further knowledge is acquired of the actual ion uptake from Hoagland solution and its variants, it is only possible to speculate as to the factors influencing carbon dioxide fixation in this experiment.

As the levels of carbon dioxide fixation occurring after pretreatment in the mineral solutions are all of the same order, and as there is no marked increase as was obtained in the distilled water pretreatment (Poel, 1952,1953), it would appear that carbon dioxide fixation is primarily determined by ion accumulation. However, the carbohydrate status may be a factor, which in turn is influenced by the presence or absence of specific elements.

In view of the results of these experiments, further investigation along the following three lines was suggested. Firstly, to determine the cause of the discrepancy in the results obtained, with those reported by Poel (1952,1953), for tap water compared with Hoagland solution pretreatment. Secondly, to study the effect of the carbohydrate status on carbon dioxide fixation, and to determine if this is a limiting factor to carbon dioxide assimilation in roots which have been pretreated in a mineral salt solution. Thirdly, to undertake a series of experiments to study carbon dioxide fixation in relation to the relative anion:cation status of the roots, using solutions of single salts from which different relative amounts of anions and cations are absorbed.

B. Tap water versus distilled water pretreatment.

As the results for Expt. 27 did not show the marked increase in carbon dioxide fixation in tap water compared with Hoagland solution pretreatment, observed by Poel (1952,1953), the experiment was repeated and the carbon dioxide uptake after distilled water pretreatment was also measured.

Root material was pretreated for 24 hours in aerated Hoagland solution, tap water, and distilled water, and was subsequently exposed to carbon-14 dioxide for a period of 1 hour. The Hoagland solution and distilled water experiments were carried out in triplicate and the tap water in duplicate. The results for the 80% ethanol soluble radioactivity and the distribution of the carbon-14 in the ethanol soluble compounds are given in Tables 27 and 28.

Table 27.

The effect of 24 hours pretreatment in a) Hoagland solution, b) distilled water, c) tap water, on carbon dioxide fixation by excised barley roots - radioactivity of the 80% ethanol soluble extracts.

Root material 0.7 gm. wet wt. Exposure medium:- 5 ml. PO_4 buffer (pH 5.6) and 200 µl. $NaHC^{14}O_3$ containing approx. 50 µc. ^{14}C and 6 µmol. CO_2. Time of exposure, 1 hour.

Pretreatment medium.	Flask No.	Activity cts/min.	Mean	s.d.	Mean for treatment	s.d.	Mean as % of control.
Hoagland	28/2	5,000					
		4,000	4,167	624			
		3,500					
Hoagland	28/4	5,000					
		5,000	4,833	745	4,667	360	
		4,500					
Hoagland	28/6	5,500					
		4,500	5,000	408			
		5,000					
Distilled water	28/8	22,500					
		23,000	22,666	235			
		22,500					
Distilled water	28/10	21,500					
		20,500	21,000	408	20,833	1,569	447.7
		21,000					
Distilled water	28/30	19,000					
		18,000	18,833	624			
		19,500					
Tap water	28/14	7,500					
		7,500	7,666	235			
		8,000					
					8,499	833	182.7
Tap water	28/16	9,500					
		9,000	9,333	236			
		9,500					

	Level of probability
a) Within treatment.	
No. 28/2 : No. 28/4 - not significant	0.4
No. 28/2 : No. 28/6 - " "	0.2
No. 28/4 : No. 28/6 - " "	0.7
No. 28/8 : No. 28/10 - significant	0.01
No. 28/8 : No. 28/30 - "	0.01
No. 28/10: No. 28/30 - "	0.05
No. 28/14: No. 28/16 - "	0.01
b) Between treatments.	
Control : Distilled water - significant	0.001
Control : Tap water - "	0.02

Table 28.

The effect of 24 hours pretreatment in a) Hoagland solution, b) distilled water, c) tap water, on carbon dioxide fixation by excised barley roots - relative activity of the labelled ethanol soluble compounds, obtained by counting chromatograms.

Treatment:-	Hoagland solution	Distilled water	Tap water
Flask No.	28/4	28/10	28/16
Compound	% activity counted		
Malic	20.0	70.5	44.5
Citric	7.2	2.0	5.1
Aspartic	14.4	3.7	3.6
Glutamic	9.6	3.7	9.5
Serine	7.2	1.7	2.2
Asparagine	6.4	4.9	8.8
Glutamine	6.4	8.3	14.6
Alanine	3.2	0.6	-
PO_4 esters	4.0	0	2.2
Unknown A.	3.2	2.9	6.6
Unknown B.	0.8	0.9	2.2
Unknown C.	4.0	0	0.7
Unknown D.	1.6	-	-
Unknown E.	1.6	-	-
Unknown F.	4.0	0.9	-
Unknown G.	6.4	-	-

- compound not present on the radioautograph.

The overall low activity was due to loss in activity of the labelled sodium bicarbonate during storage, owing to inadequate refrigeration. Subsequent carbon-14 supplies were stored in a refrigerator set at -10°C.

The results show a marked increase in the carbon dioxide uptake by the roots pretreated in distilled water over that occurring after tap water or mineral solution treatment. The distribution of the carbon-14 in the ethanol soluble products shows that this increase is in malic acid.

In the original University of California experiments (Pool 1952, 1953), tap water and distilled water pretreatments yielded the same increase in carbon dioxide fixation over Hoagland solution. An analysis of the Berkeley water supply showed it to be extremely low in mineral salts, whereas the St. Andrews supply is permanently hard. A detailed analysis of the St. Andrews tap water was not available, but the presence of those salts causing permanent hardness would render it equivalent in effect to a dilute mineral solution. Therefore, pretreatment in St. Andrews tap water would lead to a reduction in carbon dioxide uptake similar to that with Hoagland solution, as compared with distilled water pretreatment.

The difference between this experiment and No.27 in the

percentage increase of carbon dioxide fixation in tap water versus Hoagland solution pretreatment, and also the greater relative activity located in malic acid, may have arisen from changes which doubtless occur in the mineral content of tap water. Thus, "hard" tap water which may vary in its mineral salt content is not suitable as a control pretreatment medium. The use of tap water as a medium for germinating and growing the barley was changed for later experiments to distilled water, it being desirable to have roots of low salt-content.

Pool (1952, 1953) observed an increase in fixation of 500-600% in distilled water pretreatment over that with Hoagland solution. Neither in this, nor in subsequent experiments where distilled water was used as the culture medium, was there such a marked increase. The effect of the seed constituents and the nutritional history of the plants in determining the response to mineral deficiencies (Humphries, 1950; Steinberg, 1951), and in affecting nitrogen metabolism (Willis and Yemm, 1955) have been emphasised. These would perhaps account for the differences observed in carbon dioxide uptake in the two series of investigations.

C. The effect of added respiratory substrate on carbon dioxide fixation.

Ulrich (1941) observed that barley roots, which were exposed to potassium bromide solution for over 20 hours, were unable to maintain the acid-base equilibrium in the buffer system of the plant cells, owing to their inability to synthesize organic acids in the absence of a supply of available sugar.

To determine whether the reduced fixation in the presence of mineral ions was due to depletion of respiratory substrate by salt respiration, carbon dioxide fixation after pretreatment in Hoagland solution with added glucose was measured. If substrate was the limiting factor, then fixation after treatment in the mineral solution in the presence of sugar, should be of the same order as that occurring after distilled water treatment.

Root material was aerated for 24 hours in Hoagland solution + 1% glucose, and in Hoagland solution and distilled water as controls. Exposure to carbon-14 dioxide was carried out as previously described for a period of 1 hour. Each condition was duplicated. The results for the radioactivity in the 80% ethanol soluble extracts, and for the distribution of the carbon-14 in the ethanol soluble compounds are given in Tables 29 and 30.

a) Within treatment.		Level of Probability
No.29/7 : No.29/9	- not significant	0.5
No.29/13: No.29/15	- " "	0.9

b) Between treatments.

			Level of Probability
Control	: Hoagland + 1% glucose	- significant	0.01
Control	: Distilled water	- "	0.001
Hoagland + 1% glucose	: Distilled water	- "	0.001

Table 29.

The effect of added respiratory substrate on carbon dioxide fixation in excised barley roots - 24 hours pretreatment in a) Hoagland solution, b) Hoagland solution + 1% glucose, c) distilled water:- radioactivity of the 80% ethanol soluble extracts.

Root material 0.7 gm. wet wt. Exposure medium:- 5 ml. PO_4 buffer (pH 5.6) and 200 µl $NaHC^{14}O_3$ containing approx. 50 µc. O^{14} and 6 µmol. CO_2. Exposure time 1 hour.

Pretreatment medium	Flask No.	Activity cts/min.	Mean	s.d.	Mean for treatment	s.d.	Mean as % of control
Hoagland	29/1	132,000 141,000 139,000 136,000	137,000	3,391	137,000	0	
Hoagland	29/3	111,000 113,500 115,500	113,333	1,843[x]			
Hoagland + 1% glucose	29/7	157,500 154,000 163,000 160,000 163,000	159,500	3,435			
					160,375	875	117.0
Hoagland + 1% glucose	29/9	161,500 163,500 156,500 163,500	161,250	2,861			
Distilled water	29/13	353,500 353,500 345,000 356,000	352,125	4,292			
					351,412	712	256.5
Distilled water	29/15	351,000 341,500 371,500 339,500	350,700	12,680			

x - omitted from mean since the extract had to be refiltered resulting in loss of activity.

Table 30.

The effect of added respiratory substrate on carbon dioxide fixation in excised barley roots - 24 hours pretreatment in aerated a) Hoagland solution, b) Hoagland solution + 1% glucose, c) distilled water - relative activity of the labelled ethanol soluble compounds, obtained by counting chromatograms.

Treatments:-	Hoagland solution	Hoagland + 1% glucose	Distilled water
Flask No.	29/1	29/7	29/13
Compound.		% activity counted.	
Malic	26.7	22.6	73.5
Citric	5.3	9.4	1.5
Aspartic	16.6	10.4	3.4
Glutamic	12.1	26.2	2.9
Serine	7.2	1.2	0.9
Asparagine	5.0	3.9	5.2
Glutamine	18.4	17.9	9.4
Alanine	1.1	1.6	0.3
PO_4 esters	2.4	1.7	0.5
Unknown A.	4.2	4.5	1.9
Unknown B.	1.0	0.5	0.2

The results indicate that the addition of respiratory substrate to the mineral solution during the pretreatment period enhances the carbon dioxide fixation, but the increased fixation is not of the same magnitude as that following distilled water treatment. Moreover, the distribution of carbon-14 in the ethanol soluble products shows that the increased fixation with added glucose is in glutamic acid and not in malic acid as in the case of distilled water pretreatment.

The effect of glucose on barley root metabolism in the presence of mineral ions has been studied by Milthorpe and Robertson (1948). The presence of 2% glucose did not affect the salt uptake from 0.01 M potassium chloride. Laties (1947, 1949a) measured the change in the rate of oxygen uptake by excised barley roots treated with 2% glucose, in the presence of phosphate buffer as the supporting medium. He observed a 30% increase in normal roots, and an increase of 80% in starved roots.

Investigations have indicated that the presence of glucose stimulates the nitrogen metabolism of roots (Lundegårdh, 1949b; Harley et al, 1954). Lundegårdh observed the complete disappearance of nitrate ions from the terminal regions of wheat roots after treatment with glucose, which he correlated with an increased synthesis of amino acids and proteins.

Harley et al (1954) found that storage of beech mycorrhizas in glucose led to an increased absorption of ammonium salts, and to a diminished uptake of phosphate.

It is therefore suggested that the presence of glucose during pretreatment increased the ability of the roots to synthesize nitrogen compounds, as is indicated by the increased incorporation of the carbon-14 dioxide into glutamic acid. The results are of interest in relation to the mineral deficiency experiments, where it was suggested that the protein-carbohydrate status of the roots might influence not only the amount of carbon dioxide fixation, but also the compounds into which the carbon-14 enters.

Since the reduced fixation in mineral solution compared with distilled water pretreatment was still evident, it may be concluded that respiratory substrate is not the limiting factor, but that the mineral ions present during the pretreatment period largely determine the magnitude of the carbon dioxide assimilation.

D. Pretreatment in single salt solutions.

The role of the organic acids in maintaining a constant pH within the root during mineral ion absorption has been investigated by various workers. (Hoagland, 1940, 1948;

Ulrich, 1941, 1942; Overstreet et al, 1942; Lundegardh, 1946, 1949a, 1949b, 1954; Jacobson, 1950; Burstrom, 1951; Jacobson and Ordin, 1954). The influence of mineral ion pretreatment on the subsequent carbon dioxide fixation in the presence of phosphate buffer might be correlated with the changes in the organic acid content.

Jacobson and Ordin (1954) investigated the changes in the ionic balance, and the corresponding changes in the organic acid content, in excised Sacramento barley roots during absorption from 0.005 N solutions of potassium bromide, potassium bicarbonate, and calcium bromide, which provided approximately equal absorption of anions and cations, excess uptake of cations and of anions, respectively. The following experiments were carried out to determine whether the changes in the organic acid content found in response to these different single-salt solutions were related to the subsequent carbon dioxide fixation.

Root material after 24 hours aeration in 0.005 N potassium bromide, potassium bicarbonate, and calcium bromide solutions and in distilled water as control, was exposed to carbon-14 dioxide for a period of 1 hour. Each treatment was carried out in duplicate. The entire experiment was repeated to check the results obtained. As the presence of bicarbonate

ions in the pretreatment medium may have had a special effect on the subsequent carbon dioxide fixation, a further experiment was carried out to compare carbon dioxide fixation after pretreatment in 0.005 N potassium bromide, potassium bicarbonate, and potassium sulphate solutions.

The results for the radioactivity in the 80% ethanol soluble extracts are given in Tables 31, 32, and 33. The distribution of the carbon-14 in the labelled ethanol soluble compounds is shown in Tables 34, 35, and 36.

		Level of probability
a) Within treatment.		
No.35/2 : No.35/4	- not significant	0.3
No.35/6 : No.35/8	- " "	0.1
No.35/10 : No.35/30	- significant	0.001
No.35/14 : No.35/16	- "	0.02
b) Between treatments.		
Control : 0.005 N KBr	- not significant	0.4
Control : 0.005 N $KHCO_3$	- significant	0.001
Control : 0.005 N $CaBr_2$	- "	0.01

Table 31.

The effect of 24 hours pretreatment in a) distilled water, b) 0.005 N potassium bromide, c) 0.005 N potassium bicarbonate, d) 0.005 N calcium bromide on carbon dioxide fixation by excised barley roots - radioactivity of the 80% ethanol soluble extracts.

Root material 0.7 gm. wet wt. Exposure medium:- 5 ml PO_4 buffer (pH 5.6) and 200 µl $NaHC^{14}O_3$ containing approx. 50 µc. C^{14} and 4.5 µmol. CO_2. Time of exposure 1 hour.

Pretreatment medium	Flask No.	Activity cts/min.	Mean	s.d.	Mean for treatment	s.d.	Mean as % of control
Distilled water	35/2	225,500 209,500 225,000 217,500	219,375	6,521			
					223,000	3,625	
Distilled water	35/4	224,000 225,500 230,500 226,500	226,625	2,408			
0.005 N KBr	35/6	218,500 207,500 209,000 207,000	210,500	4,677			
					216,000	5,500	96.86
0.005 N KBr	35/8	212,500 227,500 217,500 228,500	221,500	6,744			
0.005 N $KHCO_3$	35/10	63,500 63,000 62,500 60,500	62,375	1,138			
					59,750	2,625	26.79
0.005 N $KHCO_3$	35/30	58,000 57,000 57,500 56,000	57,125	718			
0.005 N $CaBr_2$	35/14	318,000 317,000 303,000 320,500 312,000	314,000	6,201			
					307,513	6,486	137.90
0.005 N $CaBr_2$	35/16	304,980 296,310 298,350 304,470	301,027	3,766			

		Level of probability
a) Within treatment.		
No.36/2	: No.36/4 - significant	0.001
No.36/6	: No.36/8 - not significant	0.1
No.36/10	: No.36/30 - " "	0.1
No.36/14	: No.36/16 - significant	0.001
b) Between treatments.		
Control	: 0.005 N KBr - significant	0.05
Control	: 0.005 N $KHCO_3$ - "	0.01
Control	: 0.005 N $CaBr_2$ - not significant	0.3

Table 32.

The effect of 24 hours pretreatment in a) distilled water, b) 0.005 N potassium bromide, c) 0.005 N potassium bicarbonate, d) 0.005 N calcium bromide on carbon dioxide fixation in excised barley roots - radioactivity of the 80% ethanol soluble extracts.

Root material 0.7 gm. wet wt. Exposure medium:- 5 ml. PO_4 buffer (pH 5.6) and 200 µl. $NaHC^{14}O_3$ containing approx. 50 µc. C^{14} and 4.5 µmol. CO_2. Time of exposure 1 hour.

Pretreatment medium.	Flask No.	Activity cts/min.	Mean	s.d.	Mean for treatment	s.d.	Mean as % of control
Distilled water	36/2	299,500					
		303,500	300,875	3,379			
		296,000					
		304,500					
					284,937	15,937	
Distilled water	36/4	270,000					
		261,500	269,000	4,459			
		273,000					
		271,500					
0.005 N KBr	36/6	197,500					
		195,000					
		182,500	189,000	8,420			
		196,000					
		176,500					
					184,700	4,800	64.80
0.005 N KBr	36/8	179,000					
		188,500					
		180,500	179,900	4,810			
		174,000					
		177,500					
0.005 N $KHCO_3$	36/10	68,500					
		68,000	66,625	1,780			
		66,000					
		64,000					
					69,182	2,557	24.28
0.005 N $KHCO_3$	36/30	65,500					
		76,500	71,740	4,206			
		74,500					
		70,500					
0.005 N $CaBr_2$	36/14	415,000					
		424,500					
		416,000	418,700	5,036			
		425,000					
		413,000					
					374,250	55,500	131.30
0.005 N $CaBr_2$	36/16	329,000					
		330,000					
		326,000	329,800	2,182			
		331,000					
		332,500					

		Level of probability
a) Within treatment.		
No.37/2 : No.37/4	- significant	0.01
No.37/6 : No.37/8	- not significant	0.4
No.37/10 : No.37/30	- significant	0.001
b) Between treatments.		
0.005 N KBr : 0.005 N $KHCO_3$	- significant	0.01
0.005 N KBr : 0.005 N K_2SO_4	- not significant	0.3

Table 33.

The effect of 24 hours pretreatment in a) 0.005 N potassium bromide, b) 0.005 N potassium bicarbonate, c) 0.005 N potassium sulphate, on carbon dioxide fixation in excised barley roots - radioactivity of the 80% ethanol soluble extracts.

Root material 0.7 gm. wet wt. Exposure medium:- 5 ml. PO_4 buffer (pH 5.6) and 200 µl $NaHC^{14}O_3$ containing approx. 50 µc. C^{14} and 4.2 µmol. CO_2. Time of exposure 1 hour.

Pretreatment medium	Flask No.	Activity cts/min	Mean	s.d.	Mean for treatment	s.d.	Mean as % of control
0.005 N KBr	37/2	305,500					
		299,000	299,500	3,921			
		299,000					
		294,500					
					308,750	9,250	
0.005 N KBr	37/4	322,500					
		315,000	318,000	4,623			
		322,500					
		312,000					
0.005 N $KHCO_3$	37/6	86,000					
		83,500	87,625	3,090			
		91,500					
		89,500					
					88,437	812	28.66
0.005 N $KHCO_3$	37/6	89,500					
		88,500	89,250	829			
		90,500					
		88,500					
0.005 N K_2SO_4	37/10	248,000					
		250,500	251,750	7,385			
		244,500					
		264,000					
					271,125	19,375	89.86
0.005 N K_2SO_4	37/30	282,500					
		296,500	290,500	5,050			
		291,500					
		291,500					

Table 34.

The effect of 24 hours pretreatment in a) distilled water, b) 0.005 N potassium bromide, c) 0.005 N potassium bicarbonate, d) 0.005 N calcium bromide, on carbon dioxide fixation by excised barley roots - relative activity of the labelled ethanol soluble compounds, obtained by counting chromatograms.

Treatment:-	Distilled water	0.005 N KBr	0.005 N $KHCO_3$	0.005 N $CaBr_2$	
Flask No.	35/2	35/6	35/10	35/14	35/16
Compound	% activity counted				
Malic	81.0	65.8	54.4	59.4	61.9
Citric	0.4	1.9	2.1	1.9	2.0
Aspartic	4.3	11.6	8.5	8.5	7.5
Glutamic	5.3	14.1	13.5	11.5	10.8
Serine	1.3	1.4	5.4	1.5	1.2
Asparagine	1.4	0.7	2.2	8.3	6.3
Glutamine	3.4	1.3	5.2	5.0	5.6
Alanine	0.4	1.0	1.4	0.9	1.1
PO_4 esters	0.7	0.8	3.9	0.5	0.7
Unknown A.	1.3	0.6	2.1	2.3	2.3
Unknown B.	0.4	0.8	1.0	0.4	0.5

Table 35.

The effect of 24 hours pretreatment in a) distilled water, b) 0.005 N potassium bromide, c) 0.005 N potassium bicarbonate, d) 0.005 N calcium bromide, on carbon dioxide fixation by excised barley roots - relative activity of the labelled ethanol soluble compounds, obtained by counting chromatograms.

Treatment:-	Distilled water	0.005 N KBr	0.005 N $KHCO_3$	0.005 N $CaBr_2$
Flask No.	36/2	36/6	36/10	36/16
Compound.		% activity counted.		
Malic	97.0	70.8	71.4	81.9
Citric	0.5	0.4	2.0	0.7
Aspartic	0.2	5.8	3.4	2.1
Glutamic	0.2	2.6	7.7	3.3
Serine	0	1.6	3.6	1.1
Asparagine	0	3.5	1.6	3.5
Glutamine	0	6.6	3.9	3.3
Alanine	0	1.2	0.9	0.5
PO_4 esters	0.4	0.8	2.0	0.3
Unknown A.	0.2	5.6	3.4	3.0
Unknown B.	0.9	1.1	0.6	0.3
Unknown C.	0.4	-	-	-

Table 36.

The effect of 24 hours pretreatment in a) 0.005 N potassium bromide, b) 0.005 N potassium bicarbonate, c) 0.005 N potassium sulphate, on carbon dioxide fixation by excised barley roots - relative activity of the 80% ethanol soluble compounds, obtained by counting chromatograms.

Treatment:-	0.005 N KBr	0.005 N $KHCO_3$	0.005 N K_2SO_4	
Flask No.	37/4	37/8	37/10	37/30
Compound	% activity counted			
Malic	67.9	73.1	84.9	83.2
Citric	1.6	1.1	0.4	0.5
Aspartic	9.9	2.4	2.4	2.5
Glutamic	11.6	5.9	3.4	4.0
Serine	1.7	4.7	1.2	1.6
Asparagine	1.8	1.8	1.1	1.5
Glutamine	3.4	5.7	3.9	4.2
Alanine	0.4	1.0	0.5	0.6
PO_4 esters	0.5	1.5	0.6	0.6
Unknown A.	0.8	1.3	1.2	1.1
Unknown B.	0.3	1.3	0.2	0.2

The results of these experiments with single-salt solutions lend strong support to the hypothesis that carbon dioxide fixation in mineral element pretreatment experiments is correlated with the cation-anion ratio.

In these experiments, the carbon dioxide fixation occurs concurrently with ion absorption from the phosphate buffer. The amount of such ion absorption will be determined by the mineral status of the roots, and therefore by the form of pretreatment. Ulrich (1941) investigated ion absorption in potassium dihydrogen phosphate by excised "low-salt" barley roots, and found negligible anion accumulation after an 8 hour period, but absorption of potassium led to an increased organic acid content within the roots. Therefore, in the case of distilled water pretreatment it would be expected that the roots entering the phosphate buffer medium would have a high capacity for cation absorption; potassium accumulation would take place, coupled with an intercellular rise in the level of malic acid as demonstrated by Hoagland (1948),

Burström (1951), and Jacobson and Ordin (1954), and to an increase in carbon dioxide fixation, as reported by Poel (1952,1953), and which is confirmed in the results of the present investigation.

With exactly equal absorption of cations and anions, it might be expected that fixation of carbon-14 dioxide would be of the same order as in roots pretreated in distilled water. That such is not the case in experiments with potassium bromide would confirm that there is a slight excess of cation absorption from this salt as demonstrated by Ulrich (1941), and Jacobson and Ordin (1954).

From the calcium bromide solution mainly anion absorption should bring about a more pronounced decline in the organic acid content, than in the distilled water pretreatment. Therefore when cation accumulation takes place from the phosphate buffer, the increase in the organic acid fraction and the carbon dioxide fixation will be correspondingly greater.

In potassium bicarbonate solution, potassium

will tend to accumulate during pretreatment, with a consequent fall in carbon dioxide fixation in the following stage of the experiment. Potassium sulphate pretreatment has a similar, though not so marked effect on carbon dioxide fixation. Ulrich (1941) measured ion absorption from potassium bicarbonate and potassium sulphate and found a much greater excess of cation absorption in the former. In the case of potassium bicarbonate the reduction in the subsequent carbon dioxide fixation may be due not only to the excess cation absorption during the pretreatment, but also to the synthesis of unlabelled malate from the bicarbonate ions. Overstreet et al (1942) suggest that bicarbonate ions may only have an intermediate role as carriers in ion absorption, and their results indicate that the excess accumulation of cations over anions is roughly balanced by organic anions, other than bicarbonate, which are synthesized within the plant.

Jacobson (1955) has recently studied carbon-14 dioxide fixation in excised barley roots during actual treatment with potassium bromide, potassium dihydrogen

phosphate, and calcium bromide; this provides a direct measurement of carbon dioxide fixation in relation to mineral ion absorption, but has the disadvantage that the conditions under which the material is exposed to the carbon-14 dioxide are not controlled to the same extent as in the "indirect" method using phosphate buffer. His results also show that the magnitude of carbon dioxide fixation is largely determined by the concurrent ion absorption, and that the carbon-14 is mainly incorporated into the organic acid fraction, which maintains the anion-cation balance within the root.

In the present investigation, the data for the distribution of the carbon-14 in the ethanol soluble fraction, obtained from counting chromatograms, shows that the increase in the organic acid fraction due to potassium absorption, is not in every case reflected entirely in the malic acid, as suggested by Hoagland (1948), Burström (1951), and Jacobson and Ordin (1954). The differences in the relative activities in the carboxylic and amino acids for Expts. 35 and 36,

would suggest that differences in the nutritional and metabolic status of the roots may influence the distribution of the incorporated carbon-14. In the case of Expt. 35, 9 day-old roots were used (owing to the longer germination period required for the second batch of seed) and the seed was grown throughout in the light. In Expts. 36 and 37, the rate of germination was increased by covering the seed with enamel dishes during the first 4 days of germination and growth, and the roots for the experiments were excised from 7 and 8 day-old seedlings respectively. This may have led to a lower carbohydrate content in the roots for Expt. 36 compared to those in Expt. 35, and therefore to a reduced tendency for protein and amino acid synthesis. Jacobson (1955) investigated Sacramento barley roots which had been grown throughout in a dark incubator, and his qualitative analyses of the radioactive ethanol soluble compounds, showed glutamic acid as the only labelled nitrogen product present.

Thimann and Bonner (1950) state that the influence of cations on the organic acid content of plants is

complex; the medium in which the plants are grown influences the reaction of the organic acid fraction to potassium absorption, and moreover, changes in malic acid are not necessarily involved. Steward (1937) also observed that the relative anion:cation uptake by barley roots was determined by the metabolic state of the tissues, which in turn is regulated by the internal composition and previous nutritional history. He found that whereas calcium salts caused a decrease in respiration and protein synthesis, absorption of potassium led to an increased protein synthesis. Moreover, the presence of calcium ions has a stimulating effect on potassium absorption (Jacobson et al, 1950; Overstreet et al, 1952). It is suggested that the presence of calcium ions during the pretreatment would lead to an increased uptake of potassium during the exposure period, and consequently to an increased formation of organic acids. Moreover, in the presence of adequate sugar substrate, amino acid formation would occur, to balance the decreased protein and amino acid synthesis caused by pretreatment in the presence of calcium ions.

The high percentage of carbon-14 located in the

malic fraction after potassium sulphate treatment also deserves consideration. The presence of an immobile anion reduces cation absorption (Dale and Sutcliffe, 1956) and the uptake of potassium from potassium sulphate is considerably lower than from potassium bromide (Hoagland, 1940; Ulrich, 1941; Overstreet et al, 1942). But the absence of anion uptake from potassium sulphate solution results in the roots having a high cation and organic acid content prior to exposure to carbon-14. Consequently the organic acid formation will be low, and carbon dioxide fixation will be small during the exposure period. It is of interest that the distribution of the incorporated carbon-14 follows a similar pattern to that obtained in distilled water pretreatment. This might suggest that the anion content of the roots may also be of importance in determining the nature of the labelled products.

In conclusion it can be stated that the amount of carbon dioxide fixation appears to be determined by the relative anion:cation balance within the root tissues, and this in turn may be correlated with the increased formation of organic acids occurring in response to excess cation absorption, the organic acid content

being taken to include not only the carboxylic acids but also the amino acids. The specific acids involved are apparently governed by the previous history of the roots.

VI. The effects of respiratory inhibitors on carbon dioxide fixation.

The effect of inhibitors on the fixation of carbon dioxide was investigated to elucidate the mechanism whereby the carbon dioxide is incorporated, and to study the subsequent metabolic pathway of the fixed carbon within the root.

A. Cyanide.

The investigations of Milthorpe and Robertson (1948), Lundegårdh (1950), and Weeks and Robertson (1950), have demonstrated that cyanide inhibits salt respiration. There are variable reports regarding the terminal oxidase in barley roots. James (1953) regards ascorbic acid oxidase as the active terminal oxidase in young barley roots, and reports the complete absence of the cytochrome-cytochrome oxidase system in 6-10 day old roots. (James and Boulter, 1955). Root material for the present experiment was obtained from 10 day old seedlings. Fritz and Beevers (1955), however, state that the cytochrome-cytochrome oxidase system is in fact present in

barley roots during all stages of development, although the amount they were able to extract was insufficient to account for the total respiratory oxygen absorption. Irrespective of the relative part played by the two oxidase systems, both are sensitive to cyanide, and respiration of barley roots is strongly inhibited by treatment with cyanide.

Root material was aerated for 24 hours in Hoagland solution containing 0.001 M potassium cyanide, in 0.001 M potassium cyanide solution, and in Hoagland solution and distilled water as controls. Exposure to carbon-14 dioxide was carried out as previously described. The results for the total activity and the relative activity of the compounds present in the 80% ethanol extract are given in Tables 37 and 38.

Table 37.

The effects of pretreatment for 24 hours in a) Hoagland solution, b) Hoagland solution + 0.001 M KCN, c) Distilled water, d) 0.001 M KCN on carbon dioxide fixation by excised barley roots - radioactivity of the 80% ethanol soluble extracts.

Roots 0.7 gm. wet wt. Exposure medium:- 5 ml. PO_4 buffer (pH 5.6), 200 µl. $NaHC^{14}O_3$ containing approx. 50 µc. C^{14} and 6 µmol. CO_2. Time of exposure 1 hour.

Pretreatment medium	Flask No.	Activity cts/min.	Mean	Standard deviation	Mean as % of control.
Hoagland	31/2	91,000 92,000 92,500 92,000	91,575	545	
Hoagland + 0.001 M KCN	31/4	51,500 51,500 49,500 50,500	50,750	829	44.9
Distilled water	31/8	229,000 211,500 227,000 233,000 229,500 224,000	225,667	6,745	
0.001 M KCN	31/10	155,000 153,000 148,500 149,000 146,000 145,000	149,417	3,565	66.5

The use of the t test showed that (at P 0.001) the radioactivity for each treatment to be significantly different from that of each of the other treatments.

Table 38.

The effects of pretreatment for 24 hours in a) Hoagland solution, b) Hoagland solution+0.001 M KCN, c) Distilled water, d) 0.001 M KCN, on carbon dioxide fixation in excised barley roots - relative activity of the labelled ethanol soluble compounds, obtained by counting chromatograms.

Treatment:-	Hoagland solution	Hoagland + 0.001 M KCN	Distilled water	0.001 M KCN
Flask No.	31/2	31/4	31/8	31/10
Compound		% activity counted		
Malic	14.6	37.3	78.9	77.6
Citric	2.7	3.6	0.4	0.4
Aspartic	29.7	11.6	3.5	3.9
Glutamic	19.6	18.8	7.7	4.6
Serine	4.6	3.3	0.9	1.1
Asparagine	6.6	6.5	1.4	2.8
Glutamine	10.9	8.1	3.4	4.8
Alanine	2.1	1.4	0.6	0.6
PO_4 esters	1.0	1.3	0.3	0.5
Unknown A.	6.9	7.6	2.0	3.1
Unknown B.	0.8	0.6	0.9	0.4

The results show a decrease in the amount of carbon dioxide fixation in both cases of cyanide treatment as compared to the controls. This decrease is not reflected in any marked changes in the distribution of the carbon-14, although the Hoagland control has an unusually high activity in the aspartic acid, which does not appear in the presence of cyanide.

The values of t in every case attains significance (at P = 0.001); however, as only single experiments were run for each form of pretreatment, these values of t are a measure not only of the difference arising from the type of treatment, but also of the variation in sampling the root material.

As the presence of 0.001 M potassium cyanide largely inhibits salt accumulation in barley roots (Merry and Goddard, 1941; Machlis, 1944a; Milthorpe and Robertson, 1948), it would follow that the mineral status of the roots after the pretreatment period should be the same in both the Hoagland+ potassium cyanide and in the cyanide alone; consequently the level of carbon dioxide should be of the same order. Moreover, the relative incorporation of the carbon-14 into the various fixation products should be similar, as previous results have indicated that the carbon dioxide fixation is largely determined by the concurrent ion absorption. As the level of fixation, and the distribution of the carbon-14 after pretreatment in

Hoagland+ potassium cyanide are not the same as in potassium cyanide, it would suggest that a reversal of the cyanide inhibition had occurred during the pretreatment period. This might be due to loss of cyanide, as pretreatment was carried out in open beakers, and no precautions were taken to restrict the volatilization of the cyanide (Lundegårdh, 1949a). Alternatively, during the 24 hour period a reorganization of the oxidase-cyanide complex might occur, which is postulated for the recovery of wheat roots from 0.0001 M potassium cyanide inhibition of salt accumulation after one to sevefal hours (Lundegårdh, 1950).

It is evident from the results that there is a more marked effect of the cyanide in the mineral solution than in the distilled water pretreatment. Differences in the pH values of the pretreatment media may have been important here:-

	Initial pH	Final pH
Hoagland + 0.001 M KCN.	7.24	6.46
0.001 M KCN.	9.45	6.89

Lundegårdh (1949a) investigated the action of inhibitors as related to the concentration of the

undissociated molecules, and his findings supported the assumption that weak acids are absorbed primarily as molecules. This accounted for the much stronger inhibitory effect of a mixture of equal quantities of potassium cyanide and hydrochloric acid (pH 7.0) compared with that of potassium cyanide alone (pH 9.6). In the present experiment the pH values of the pretreatment media were such that the absorption of the cyanide would be much greater in the Hoagland solution than in the potassium cyanide solution (pk HCN = 9.1). It may be that the increased accumulation of potassium during the pretreatment in the presence of potassium cyanide, particularly if hydrolysis to hydrogen cyanide occurs, might lead to a reduction in the capacity for absorption from the phosphate buffer, and therefore to a decrease in the carbon dioxide fixation. This effect would be more marked in the Hoagland treatment, as there would be potassium absorption not only from the mineral solution, but also perhaps an additional uptake from the potassium cyanide.

Ordin and Jacobson (1955) investigated the effect of cyanide inhibition of Atlas barley roots

on the subsequent salt absorption from potassium bromide solution, and the oxygen uptake in distilled water. The results indicated that the cyanide inhibition had a marked effect on the subsequent metabolic activity of the roots, which persisted during the 3½ hour period of measurement, leading to a reduction to 63.5% and 50.5% for potassium and bromide absorption, while the subsequent oxygen uptake was 70.5% that of the control.

If cyanide inhibition did occur in the present experiment, the effect could be reflected in a reduced uptake of potassium ions from the phosphate buffer during the exposure period, resulting in a decreased carbon dioxide fixation in response to the lower organic acid content required to maintain the anion-cation balance within the root. As 30%-40% of the salt accumulation in barley roots is not inhibited by cyanide (Merry and Goddard, 1941; Machlis, 1944a; Milthorpe and Robertson, 1948), a certain accumulation would occur during the Hoagland potassium cyanide pretreatment, and this would result in a further decrease of potassium accumulation from the phosphate buffer.

Under the experimental conditions used, no conclusions can be drawn as to the effect of cyanide on carbon dioxide fixation. A more direct method whereby carbon dioxide fixation, and changes in ion absorption and oxygen uptake could be measured simultaneously in the presence of cyanide, would be much more satisfactory for determining the role of carbon dioxide fixation in relation to ion absorption.

B. Malonate.

The effect of malonate on carbon dioxide fixation was investigated to determine the metabolic pathway of the fixed carbon-14 dioxide. Malonate is generally regarded as being a specific inhibitory of succinic dehydrogenase. Its role is that of a competitive inhibitor and Lundegårdh (1953) has demonstrated its specific effect in depressing the oxidation-reduction level of cytochrome b, which is linked to the succinic dehydrogenase system. A similar relationship of the ascorbic acid oxidase system has not yet been demonstrated (James, 1954).

Malonate inhibition of succinic dehydrogenase blocks the tricarboxylic acid cycle, and therefore

accumulation of labelled succinate would indicate that the carbon-14 dioxide is oxidatively incorporated into the plant metabolism.

The experimental procedure followed differed from the cyanide experiment in that the exposure to carbon-14 dioxide was carried out in the presence of the inhibitor. Root material was aerated for 24 hours in distilled water, and exposed to carbon-14 either in phosphate buffer (pH 5.59), or in phosphate buffer containing 0.01 M sodium malonate (pH 5.77). Each treatment was carried out in duplicate. Results for the total activity, and the relative activity of the ethanol-soluble compounds are given in Tables 39 and 40.

Table 39.

The effect of 0.01 M sodium malonate on carbon dioxide fixation by excised barley roots - radioactivity of the 80% ethanol soluble extract.

Roots 0.7 gm. wet wt. 24 hours pretreatment in aerated distilled water. Exposure medium either a) 5 ml. PO_4 buffer (pH 5.59); or b) 5 ml. PO_4 buffer containing 0.01 M Na malonate, with 200 µl. $NAHO^{14}O_3$ containing 50 µc. C^{14} and 4.2 µmol. CO_2. Time of exposure 1 hour.

Exposure medium	Flask No.	Activity cts/min.	Mean	s.d.	Mean for treatment	s.d.	Mean as % of control
PO_4 buffer	38/10	269,500 282,500 275,000 273,000	275,000	4,756			
					277,875	2,875	
PO_4 buffer	38/30	268,000 285,000 286,000 284,000	280,750	7,329			
PO_4 buffer + 0.01 M Na malonate.	38/14	218,500 214,500 206,000 212,000	212,750	4,535			
					184,625	28,125	66.4
PO_4 buffer + 0.01 M Na malonate	38/29	158,000 159,000 157,500 151,500	156,500	2,937			

	Level of probability
a) Within treatment.	
No.38/10 : No.38/30 - not significant	0.4
No.38/14 : No.38/29 - significant	0.001
b) Between treatments.	
Control : malonate - not significant	0.1

Table 40.

The effect of 0.01 M sodium malonate on carbon dioxide fixation by excised barley roots - relative activity of the labelled ethanol soluble compounds, obtained by counting chromatograms.

Treatments:-	PO_4 buffer (control)	PO_4 buffer+0.01 M Na malonate.	PO_4 buffer+0.01 M Na malonate
Flask No.	38/10	38/14	38/29
Compound.		% activity counted.	
Malic	84.9	84.9	83.7
Citric	0.6	0.6	0.3
Aspartic	3.6	2.8	3.7
Glutamic	4.2	4.6	4.0
Serine	0.9	1.3	1.1
Asparagine	1.6	1.9	2.3
Glutamine	2.9	2.5	3.7
Unknown A.	0.5	0.6	0.5
Unknown B.	0.4	0.3	0.2
Unknown C.	0.2	0.4	0.4

Interest in the malonate inhibition experiments lies mainly in the qualitative results. The radioautographs showed not only a similar range of labelled compounds in the malonate-treated roots as in the controls, but also a similar distribution of the carbon-14 with most of the activity located in the malic acid and only small quantities present as Unknown C, which was considered to be succinic acid. These results contrasted to those of Laties (1947), who found that malonate inhibition of barley roots resulted in an accumulation of succinate, and that the fixed carbon-14 dioxide was largely incorporated in the succinate fraction. Increase in the succinate level due to malonate inhibition has also been demonstrated by Bonner (1948) and Ordin and Jacobson (1955). Hanly et al. (1952) suggested that failure to demonstrate succinate accumulation was due to the competitive inhibition of malonate, and that accumulation of malonate would result in a reversal of inhibition. However Ordin and Jacobson (1955) found that 0.04 M succinate treatment of malonate-inhibited roots enhanced the inhibition of ion absorption.

Other work on the effect of malonate as an inhibitor has given divergent results. Certain

investigations have shown that malonate may also inhibit other oxidation reactions (Das, 1937; Pardee and Potter, 1949; Krebs et al, 1952; Beevers, 1952; Price, 1953). Results for the addition of organic acids in modifying the effect of malonate are also varied. (Machlis, 1944a; Bonner and Wildman, 1946; Laties, 1949a, 1949b; Hanly et al, 1951; Ordin and Jacobson, 1955). Moreover, the essential role of succinic dehydrogenase is questionable from the results of Evans (1941), Berger and Avery (1944), and Lundegardh (1949a), but Price and Thimann (1951) have suggested that inability to demonstrate succinic dehydrogenase in plant tissues may be due to its being much less stable than that obtained from animal tissues.

The importance of the pH in the study of malonate inhibition has been emphasised by Bonner and Wildman (1946), Bonner (1948), Lundegardh (1949a), and Hanly et al (1952). Inhibition is related to the entrance of undissociated molecules into the cell (Beevers, 1952), and whereas malonate is strongly inhibitory at pH 4.5, it is only slightly so at pH 5.5, and is

ineffective at pH 6.5. Failure to recognise this factor may account for the negative outcome of efforts to demonstrate succinic dehydrogenase. The inhibitory effect of malonate at pH 4.5 is the same whether it is supplied in phosphate buffer, or in water (Manly et al, 1952).

In the present investigation the pH of the supporting medium during malonate inhibition was 5.77. Therefore it is probable that the succinic dehydrogenase was at most only partially inhibited; this might account for the failure to demonstrate the accumulation of labelled succinate, observed by Latios (1947) working at pH 5.0. That the inhibition was only partially effective is also suggested by the results of Ordin and Jacobson (1955); in excised Atlas barley roots exposure for 1 hour to 0.01 M malonate was followed by a subsequent decrease in ion absorption to 8.4% and 29.7% for potassium and bromide respectively, whereas oxygen uptake was reduced to 45%. However direct comparison of results may be misleading, unless uniform material is used which has been grown and treated under specified conditions, particularly as the effect of malonate

is influenced by the mineral status of the material (Bonner, 1948).

The experiment has been repeated by Poel (unpublished data) under optimum conditions for malonate inhibition of succinic dehydrogenase. Root material was exposed to carbon-14 dioxide in 0.01 M potassium dihydrogen phosphate (pH 4.5) as control, and in 0.01 M potassium dihydrogen phosphate containing 0.01 M sodium malonate adjusted to pH 4.5 with 2 N hydrochloric acid. Results for the radioactivity in the ethanol-soluble products showed that fixation in the inhibited roots was reduced to 15.5% of that in the controls, indicating a greater inhibition than at pH 5.77. The nature and relative activity of the labelled ethanol-soluble compounds were similar to those found in the present investigation. The results of these experiments would suggest that the fixed carbon-14 dioxide may follow alternate pathways of metabolism within the root. These may involve 1) carboxylation of pyruvic and/or α-ketoglutaric acids, leading oxidatively to labelling of Krebs cycle acids as far as succinic, and 2) the reductive formation of malic acid.

Poel (1953) has shown that carbon dioxide fixation in barley roots is dependent upon aerobic conditions. As malonate decreases both the salt absorption and the oxygen uptake in barley roots (Machlis, 1944a; Ordin and Jacobson, 1955), the observed decrease in carbon-14 dioxide fixation in the presence of malonate, could be correlated with either or both of these processes.

VII. Discussion.

The various experiments demonstrate that carbon dioxide fixation is closely related to the metabolic activities of the root and, in particular, to the concurrent ion absorption. The amount of carbon dioxide assimilation appears to be determined by the relative anion:cation uptake, and differences in the magnitude of fixation are largely proportional to the labelled malic acid present. As exposure to carbon-14 dioxide was carried out in phosphate buffer, the tendency would be for excess cation accumulation to occur. The level of potassium accumulation during the exposure period would depend on the initial mineral ion status of the root material, determined by the form of pretreatment. The single salt experiments are consistent with this hypothesis.

The mineral-deficiency experiments strongly suggest that the decreased fixation after pretreatment in a mineral solution is not related to the presence of a specific element, but rather to mineral ion absorption as a whole.

Further circumstantial evidence that carbon dioxide fixation is mainly determined by the concurrent ion absorption, is indicated by the initial rapid rate of assimilation observed by Poel (1952), which may be correlated with the time course uptake of salts reported by Milthorpe and Robertson (1948).

If carbon dioxide fixation is only related to the formation of organic acids in order to maintain the anion:cation balance within the cells, then negligible fixation should occur under conditions of excess anion absorption. Jacobson (1955) has shown that assimilation does in fact occur in the presence of calcium bromide, although a reduction in both the total organic acid and malic acid fractions takes place. This fixation may be due to an exchange reaction, similar to that demonstrated by Goldberg and Sanadi (1952).

Experiments with an exogenous supply of glucose as respiratory substrate show that carbon dioxide fixation is not entirely determined by mineral ion absorption. The addition of glucose to the

pretreatment medium results in a greater formation of labelled amino acids which in turn requires an increased formation of carboxylic acids. Labelled glutamic acid is formed in all the experiments, and will be derived from labelled ∝-ketoglutarate. However, ∝-ketoglutarate is absent in the majority of the radioautographs, a feature also evident in the results of Jacobson (1955), which indicates that it has a very rapid turn-over.

The data suggest that carbon dioxide assimilation is largely influenced by the demand for carboxylation products during metabolism, and therefore fixation will be dependent on the normal operation of the oxidative organic acid cycle for the supply of those organic acids which undergo carboxylation.

The enzymatic mechanisms of carbon dioxide fixation have been discussed by various workers (Werkman and Wood, 1942; Wood and Lorber, 1949; Wood, 1951; Utter and Wood, 1951; Ochoa, 1951, 1952; Vennesland and Conn, 1952). In general dicarboxylic acids are produced by the action of

the "malic enzyme" or by oxalacetic carboxylase, resulting in the formation of malate or oxalacetate from pyruvate. The carboxylative formation of tricarboxylic acids occurs by the action of the "isocitric enzyme", yielding isocitrate from ∝-ketoglutarate. The synthesis of both labelled dicarboxylic and tricarboxylic acids, after carbon-14 dioxide fixation in parsley root preparations, has been observed by Gollub and Vennesland (1947) and Ceithaml and Vennesland (1949), and has also been demonstrated in bean roots (Kursanov et al, 1953). No data are available for the carboxylation mechanisms in barley roots. The nature of the labelled products gives no indication of the mechanisms involved, as they may all be derived in a very short time from the product of any one of the possible carboxylation reactions.

An attempt to determine whether the change in concentration of the carboxylation products corresponds to the amount of carbon-14 dioxide fixed, would be complicated by the reincorporation of endogenous

carbon dioxide. This fixation of the respiratory carbon dioxide probably accounts for the low percentage fixation of the applied carbon-14 dioxide, even under conditions of optimal malate formation (Jacobson, 1955). Respiratory quotient values during salt absorption (Ulrich, 1941; Machlis, 1944a) showed a marked correlation with the changes in the organic acid content. As these changes are reflected mainly in the malic acid fraction, decrease in the carbon dioxide output would not be evident if the malic acid were formed via the organic acid cycle, as all the decarboxylation reactions occur prior to malic acid formation. Consequently the decreased carbon dioxide output would suggest the occurrence of carboxylation reactions.

It is postulated that the presence of carbon dioxide may be essential in the root metabolism of higher plants, for the synthesis of certain dicarboxylic acids, which function not only as respiratory catalysts, but also in maintaining the internal equilibrium of the cells during ion absorption.

VIII. Summary.

Carbon dioxide fixation in excised barley roots has been investigated, particularly in relation to mineral ion absorption, using carbon-14 dioxide and radiochromatography.

As unsatisfactory results were obtained in the determination of radioactivity in the ethanol-insoluble root residues, the ethanol-soluble activity was taken as the basis for carbon-14 assimilation.

A linear zonation of fixation was demonstrated within the roots, the greatest uptake occurring in the region of the spices. Maintaining the root material in distilled water, rather than a nutrient solution, prior to exposure to carbon-14 dioxide, led to a marked increase in the carbon-14 incorporated in the ethanol-soluble fraction. This increase was principally in malic acid. Mineral deficiency experiments, involving the omission of potassium, nitrogen, and phosphorus in turn from the pretreatment medium, resulted in differences in the level of

carbon dioxide fixation compared with that for the complete mineral solution. Results for the radioactivity of the 80% ethanol extracts expressed as percentages of that found in the complete mineral solution were 70.6, 137.33, and 96.11 respectively.

Addition of glucose to the mineral solution resulted in a small increase in the level of carbon dioxide assimilation. The increase was in glutamic acid.

Experiments using single salt solutions provide support for the hypothesis that carbon dioxide fixation is largely determined by organic acid formation in response to excess cation absorption. It is suggested that the relative activity of the labelled products may be related to the metabolic status of the roots.

Malonate reduced carbon dioxide fixation, but the same range of labelled ethanol-soluble compounds was formed as in uninhibited roots, and there was no accumulation of labelled succinate.

An experiment with cyanide was inconclusive, due to unsatisfactory experimental conditions.

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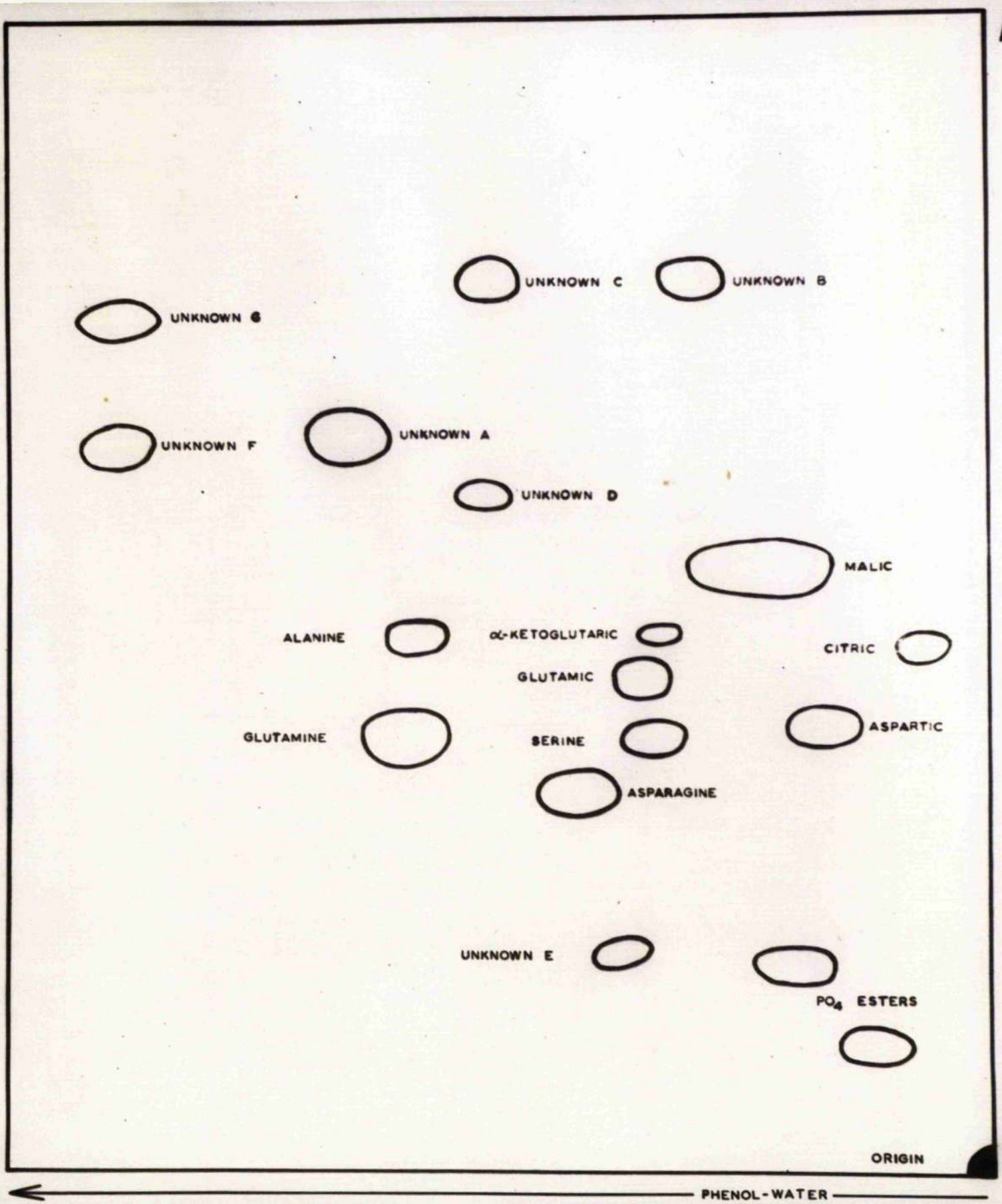
Illustrations.

Fig. 1. Location of labelled compounds (compiled from radioautographs).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UNKNOWN D
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CITRIC
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GLUTAMINE
SERINE
ASPARTIC
ASPARAGINE
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PO4 ESTERS
ORIGIN
BUTANOL - PROPIONIC ACID - WATER
PHENOL - WATER

Fig. 1.

Fig. 2. Barker's apparatus.

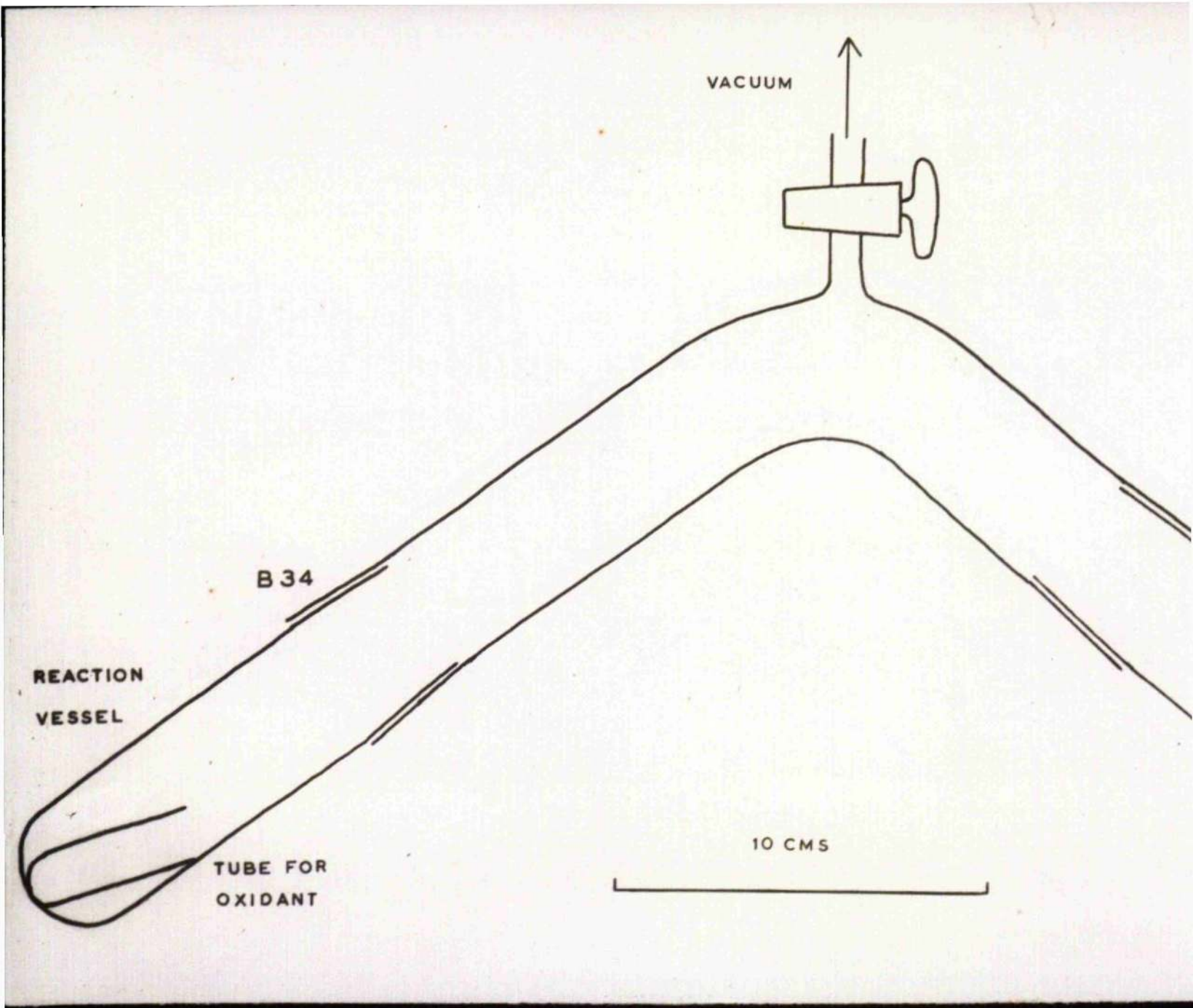


Fig. 2.

Fig. 3. Micro-apparatus No.1.

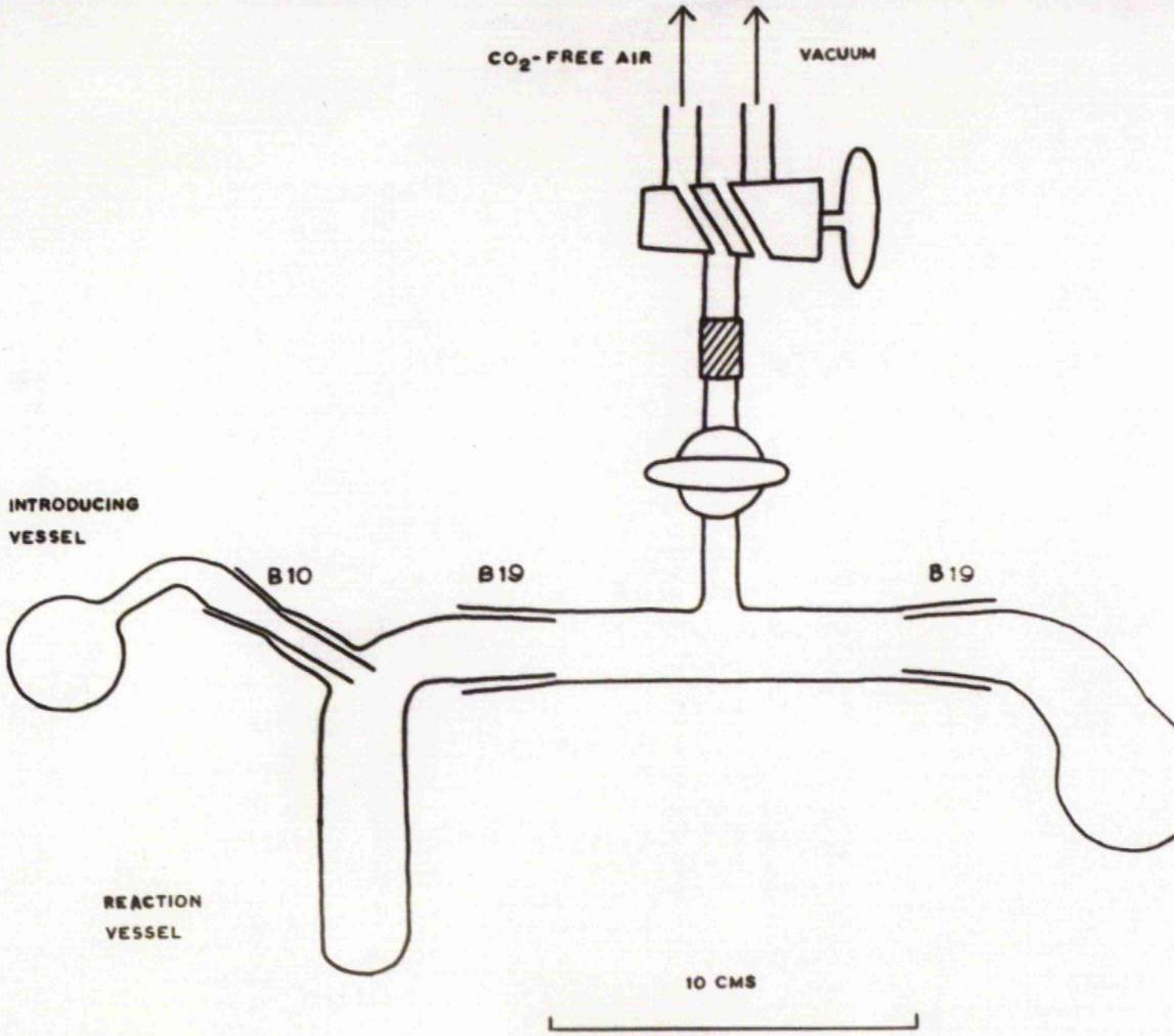

CO_2-FREE AIR
VACUUM
INTRODUCING
VESSEL
B10
B19
B19
REACTION
VESSEL
10 CMS

Fig. 3.

Fig. 4. Constant volume delivery burette.

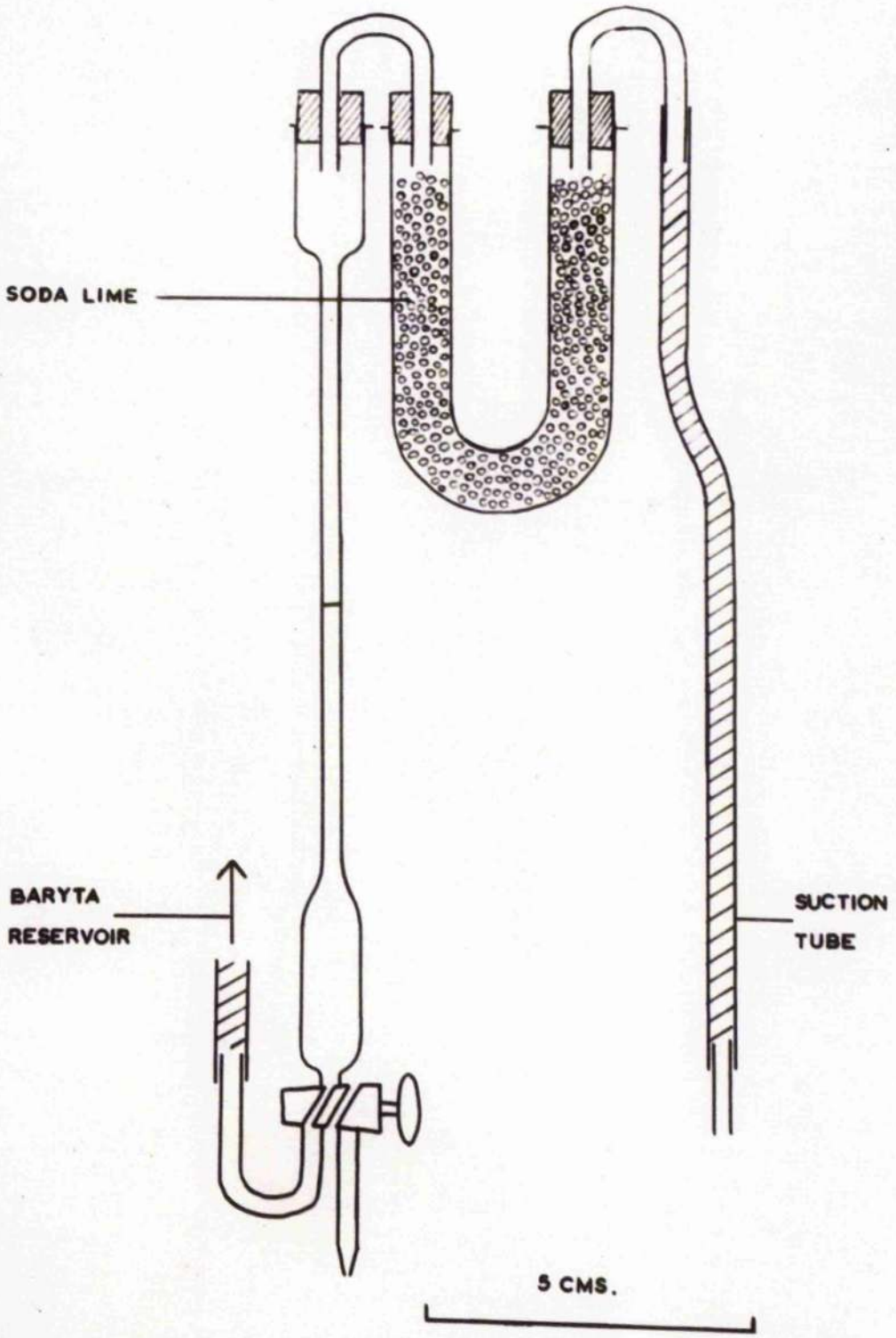
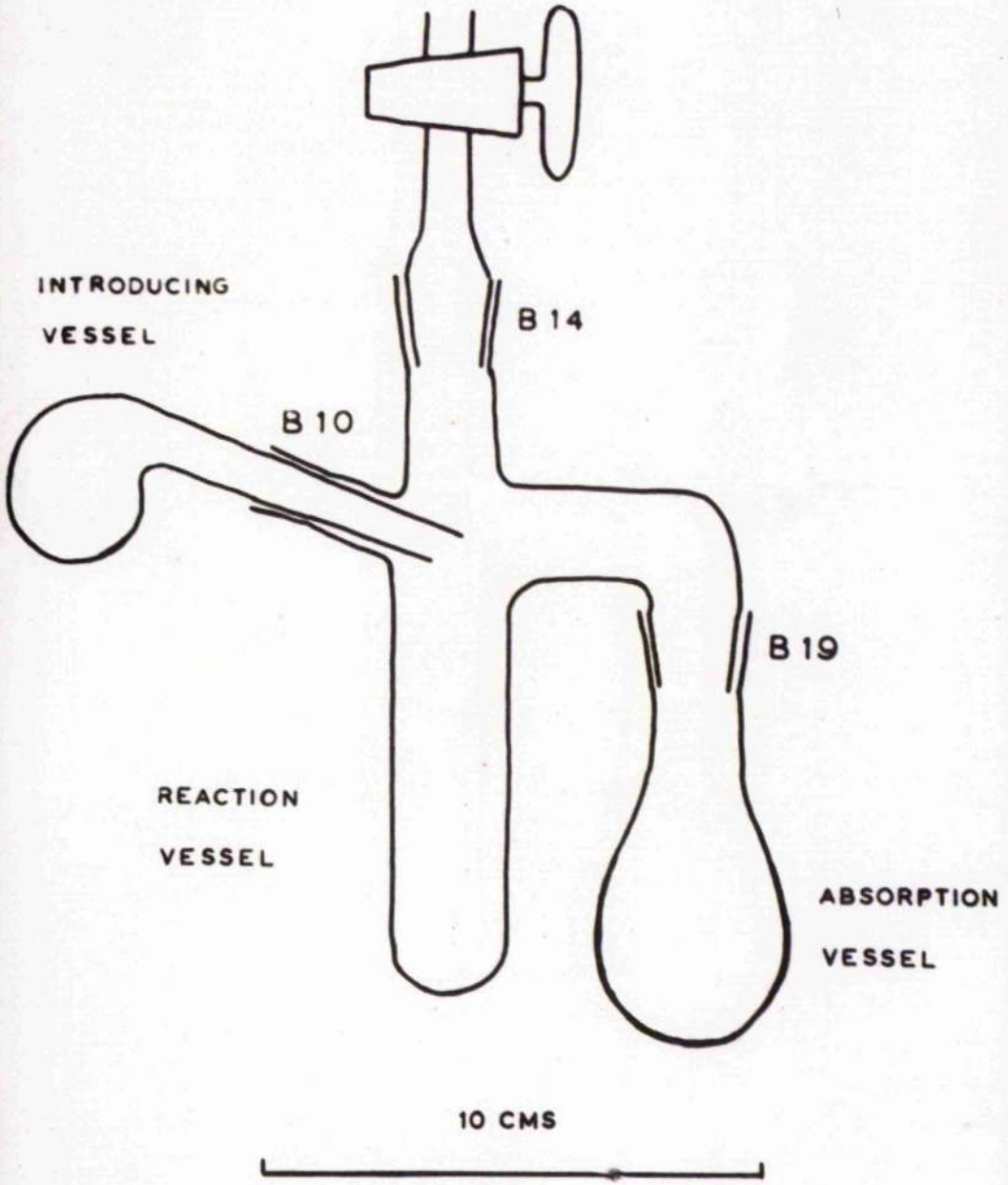


Fig. 4.

Fig. 5. Micro-apparatus No.2.


INTRODUCING
VESSEL
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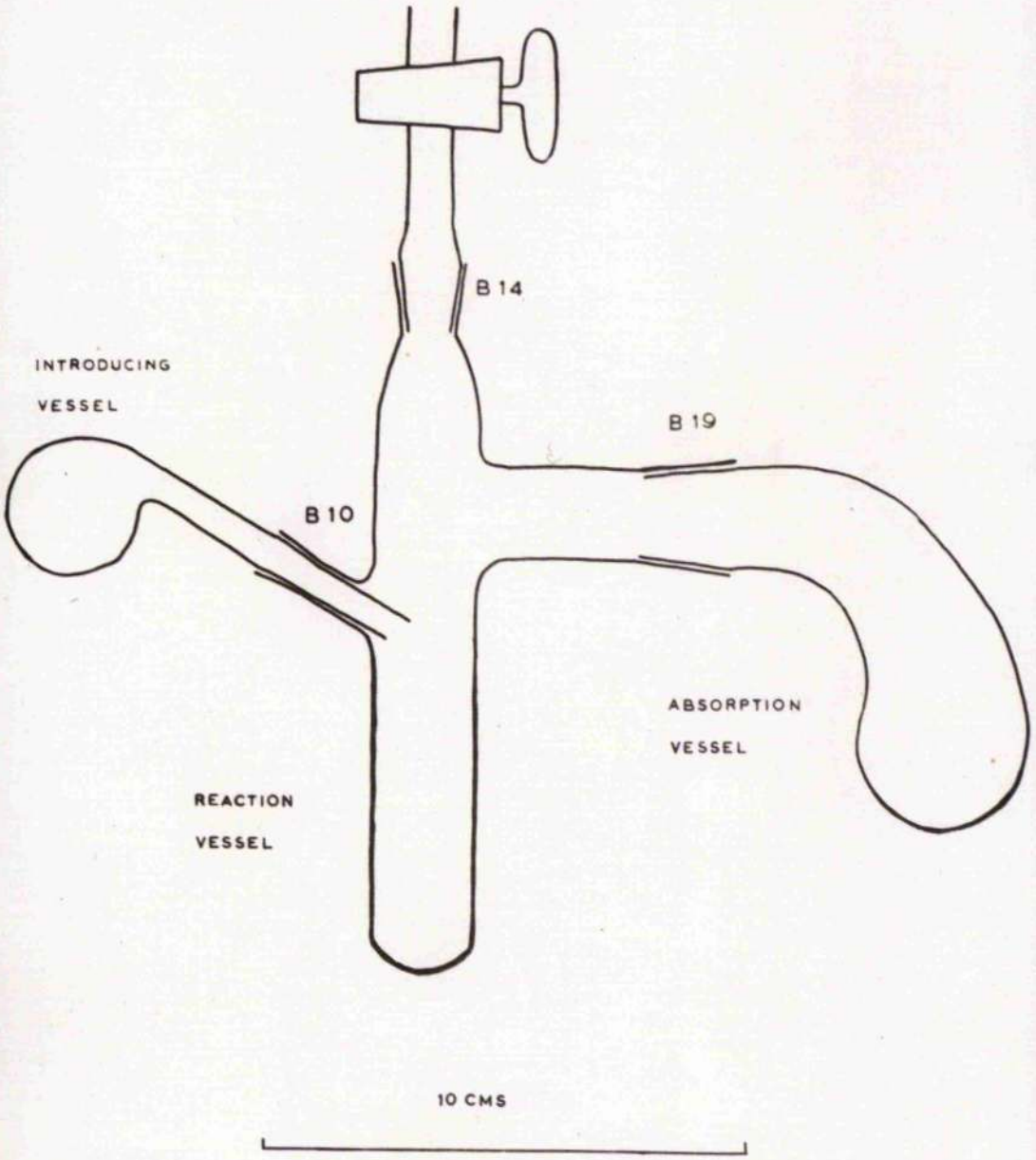
B 19
REACTION
VESSEL
ABSORPTION
VESSEL
10 CMS

Fig. 5.

Fig. 6. Modified form of Micro-apparatus No.2.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B 19
B 10
ABSORPTION
VESSEL
REACTION
VESSEL
10 CMS

Fig. 6.

Fig. 7. Graphs of the readings near the end-point in the electro-titration of barium hydroxide with added barium carbonate using -

a) 0.1 N Hydrochloric acid with phenolphthalein as indicator.

b) 0.1 N Hydrochloric acid with Simpson's mixed indicator.

c) 0.01 N Hydrochloric acid with phenolphthalein as indicator.

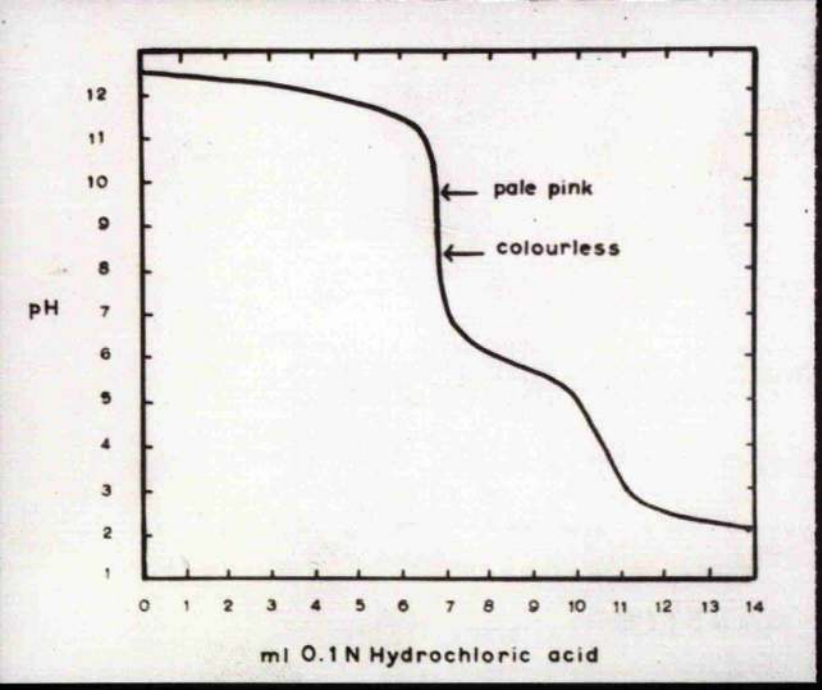

Fig. 7 a).

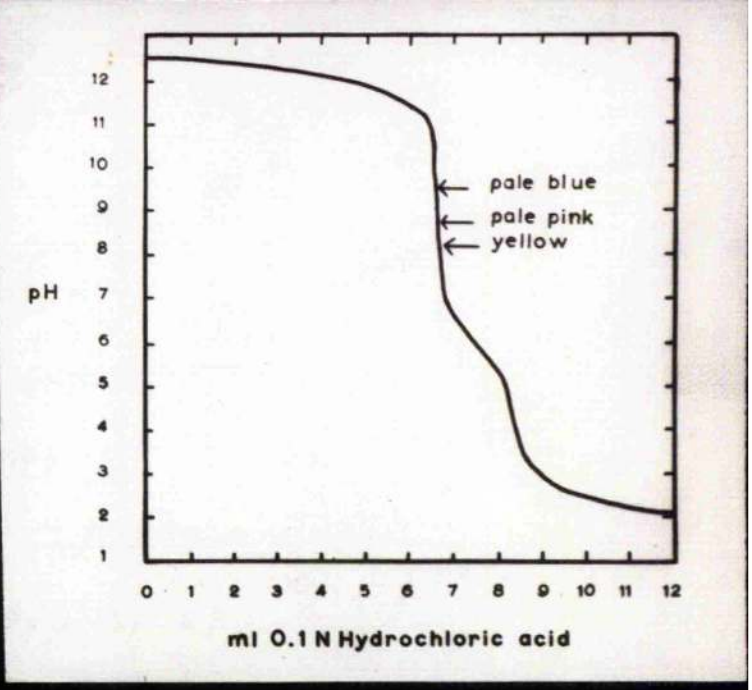

Fig. 7 b).

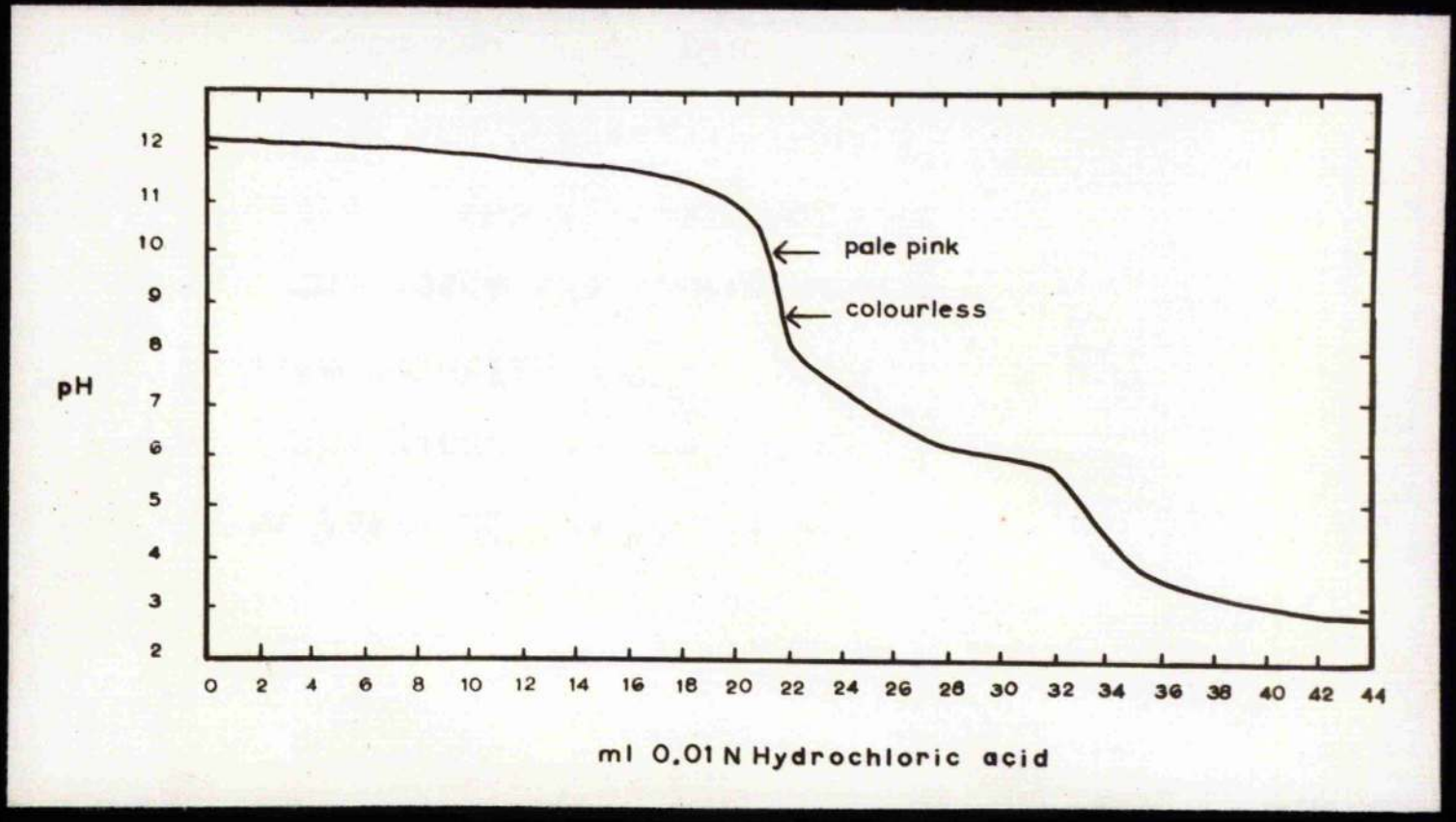

Fig. 7 c).

Fig. 8. Apparatus used in the recovery of barium carbonate by filtration.

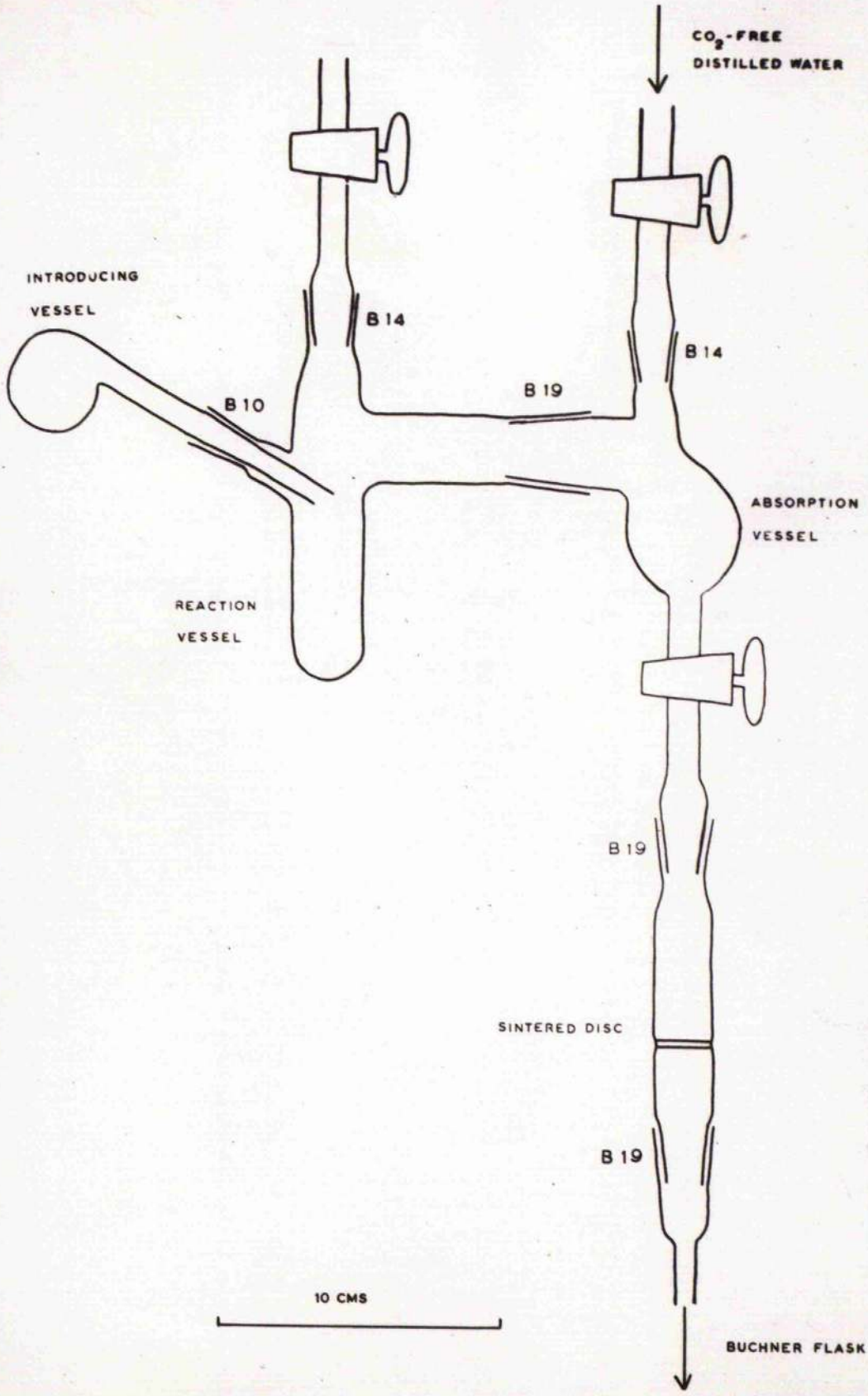
CO_2-FREE
DISTILLED WATER
INTRODUCING
VESSEL
B 14
B 14
B 10
B 19
ABSORPTION
VESSEL
REACTION
VESSEL
B 19
SINTERED DISC
B 19
10 CMS
BUCHNER FLASK

Fig. 8.